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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
This IDEA award is supporting our efforts to elucidate the roles that the ErbB4 receptor tyrosine kinase plays in breast cancer. We have generated three constitutively active mutants of ErbB4. Unlike constitutively active ErbB1 or ErbB2 mutants, the constitutively active ErbB4 mutants DO NOT couple to increased cell proliferation, to anchorage independent growth, or to a loss of contact inhibition in rodent fibroblast cell lines. Moreover, at least one of the constitutively active ErbB4 mutants is coupled to inhibition of colony formation in human mammary (tumor) cell lines and to inhibition of mammary (tumor) cell line proliferation. This suggests that ErbB4 is coupled to mammary tumor suppression in vivo.

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Table of Contents

Introduction

This IDEA award is partially supporting our efforts to elucidate the role of ErbB4 in mammary tumorigenesis. Our goals are to (1) generate a constitutively-active ErbB4 mutant; (2) assess whether the constitutively-active ErbB4 mutant inhibits the proliferation of human mammary cell lines; (3) assess whether the constitutively-active ErbB4 mutant malignantly transforms the growth of human mammary cell lines; (4) generate ErbB4 double mutants in the context of the constitutively active mutant that are either deficient for kinase activity or contain mutations at tyrosine phosphorylation sites; (5) assess whether ErbB4 kinase activity or specific ErbB4 tyrosine phosphorylation sites are critical for coupling to biological activity.

Report Body

1. Generate a constitutively-active ErbB4 mutant. The major goals of the proposed research are to generate a constitutively-active ErbB4 mutant and to use this mutant to probe ErbB4 function. We attempted to construct a constitutively-active ErbB4 mutant by deleting the extracellular ligand-binding domain. However, we could not stably express this ErbB4 mutant. We attempted to construct a constitutively-active ErbB4 mutant by replacing the transmembrane domain of human wild-type ErbB4 with the transmembrane domain of a constitutively-active rat ErbB2/HER2/Neu mutant. However, we could not stably express this ErbB2-ErbB4 chimeric protein.

Our third attempt to generate a constitutively-active ErbB4 mutant involved introducing single cysteine substitutions at five different locations in the ErbB4 extracellular juxtamembrane domain. Of these five ErbB4 mutants, three (Q646C, H647C, A648C) appear to be constitutively active for signaling; they exhibit more tyrosine phosphorylation in the absence of ligand than does wild-type ErbB4 (Figure 1) and they exhibit greater kinase activity in the absence of ligand than does wild-type ErbB4 (Figure 2).

These mutants do not malignantly transform the growth of rodent fibroblast cell lines. They do not induce foci in an assay for loss of contact inhibition (Figure 3). They do not induce growth in semi-solid medium in an assay for anchorage independence (Figure 4). Finally, they do not stimulate the growth rate or saturation density (Figure 5).

2. Assess whether the constitutively-active ErbB4 mutant inhibits the proliferation of human mammary cell lines. We have been infecting MCF-10A human mammary epithelial cells with recombinant retroviruses that express the ErbB4 mutants as well as a selectable marker (the neomycin resistance gene, which confers resistance to the antibiotic G418). We are also infecting cells with control recombinant retroviruses that contain the neomycin resistance gene alone (vector control), or the neomycin resistance gene along with wild-type ErbB4 or a constitutively-active ErbB2 mutant.

MCF-10A cells infected with the recombinant retrovirus that carries the Q646C ErbB4 mutant form far fewer drug-resistant colonies than MCF-10A cells infected with the other retroviruses (Figure 6). We infected C127 cells with the various recombinant retroviruses and quantified the number of drug-resistant colonies that resulted to permit us to account for possible differences in absolute viral titer. Even after accounting for differences in absolute viral titer, infection of MCF-10A cells with the retrovirus that contains the Q646C ErbB4 mutant results in far fewer drug-resistant colonies than expected (Table 1). This suggests that the Q646C constitutively-active ErbB4 mutant is coupled to inhibition of proliferation or induction of apoptosis in MCF-10A cells.

We have infected a panel of human mammary (tumor) cell lines (MCF-7, MCF-10A, MDA-MB-231, MDA-MB-453, SKBR-3, and T47-D) with the various recombinant retroviruses and generated stable cell lines. We are currently assessing whether the Q646C constitutively-active ErbB4 mutant or either of the other two constitutively-active ErbB4 mutants affects mammary cell growth rates or saturation densities. These experiments will enable us to assess whether ErbB4 signaling is coupled to changes in mammary cell proliferation.

Report Body

3. Assess whether the constitutively-active ErbB4 mutant malignantly transforms the growth of human mammary cell lines. Using the panel of cell lines described above we will assess whether ErbB4 signaling is coupled to changes in anchorage-independent growth. These experiments will enable us to assess whether ErbB4 signaling is coupled to malignant growth transformation or to suppression of the transformed phenotype.

4. Generate ErbB4 double mutants in the context of the constitutively active mutant that are either deficient for kinase activity or contain mutations at tyrosine phosphorylation sites. Construction of these double mutants is under way.

5. Using the ErbB4 double mutants, assess whether ErbB4 kinase activity or specific ErbB4 tyrosine phosphorylation sites are critical for coupling to biological activity. We will assay the biological activities of the ErbB4 double mutants once they have been constructed.

Key Research Accomplishments

Task 1

- Generated a number of putative ErbB4 mutants.
- Identified three ErbB4 mutants that exhibited ligand-independent tyrosine phosphorylation and increased tyrosine kinase activity.
- Determined that the three constitutively-active ErbB4 mutants do not couple to malignant growth transformation in fibroblast cell lines.

Task 2

- Generated preliminary data suggesting that the constitutively-active Q646C ErbB4 mutant inhibits drug-resistant colony formation by MCF-10A human mammary epithelial cells.
- Generated a panel of human mammary (tumor) cell lines that express wild-type ErbB4, the constitutively-active ErbB4 mutants, a constitutively-active ErbB2 mutant, or the vector control.
- Using the cell lines described above we will determine whether the constitutively-active ErbB4 mutants cause a decrease in saturation density or growth rate.

Task 3

- No progress has been made.

Task 4

- We are generating the desired ErbB4 double mutants.

Task 5

- No progress has been made.

Reportable Outcomes

- We prepared a manuscript that describes the construction and analysis of our constitutively-active ErbB4 mutants. A copy of the manuscript draft is included in the appendix of this report (Penington, *et al*).
- Mr. Desi Penington wrote and successfully defended a master's degree thesis entitled "Construction and analysis of constitutively-active mutants of the ErbB4 receptor tyrosine kinase" that is based on the results of the studies described in Task 2. A copy of Mr. Penington's thesis is included in the appendix of this report. Mr. Penington will receive his M.S. in August 2001.
- We submitted a pending grant application to the USAMRMC PCRP for additional funding to support our efforts to analyze ErbB4 function in prostate cancer cells (Dr. David J. Riese II, PI).
- We were awarded an undergraduate research fellowship by the American Association of Colleges of Pharmacy to support our efforts to analyze ErbB4 function in prostate cancer cells (Mr. Eric Williams, PI; Dr. David J. Riese II , mentor).
- We submitted a pending postdoctoral fellowship application to the USAMRMC BCRP for additional funding to support our efforts to analyze ErbB4 function in breast cancer cells (Dr. Shruti Shukla, PI; Dr. David J. Riese II, mentor).
- We were awarded an undergraduate research fellowship by the American Society for Microbiology to support our efforts to analyze ErbB4 function in breast and prostate cancer cells (Ms. Ianthe Bryant, PI; Dr. David J. Riese II, mentor).

Conclusions

We have made significant progress on the proposed research plan. We have generated three constitutively-active ErbB4 mutants. These mutants do not malignantly transform the growth of fibroblasts. Indeed, we have preliminary evidence that one of these mutants is coupled to inhibition of drug-resistant colony formation in a breast cell line. This suggests that ErbB4 may act as a tumor suppressor gene in the mammary epithelium. Furthermore experiments are underway to test this hypothesis. Experiments are also underway to genetically identify the biochemical functions of ErbB4 that are required to couple this receptor to biological responses.

References

None

Appendices: List of Documents (107 pages total)

1. Unpublished Figure 1. (1 page)
2. Unpublished Figure 2. (1 page)
3. Unpublished Figure 3. (1 page)
4. Unpublished Figure 4. (1 page)
5. Unpublished Figure 5. (1 page)
6. Unpublished Figure 6. (1 page)
7. Unpublished Table 1. (1 page)
8. Penington D.J., Bryant I., and Riese D.J. II. "Constitutively-Active ErbB4 and ErbB2 Mutants Exhibit Distinct Biological Activities." For submission to *Cell Growth and Differentiation* (35 pages).
9. "Construction and Analysis of Constitutively-Active Mutants of the ErbB4 Receptor Tyrosine Kinase", M.S. Thesis, Mr. Desi Penington, July 2001 (65 pages).

Figure 1. Three ErbB4 mutants exhibit constitutive tyrosine phosphorylation.

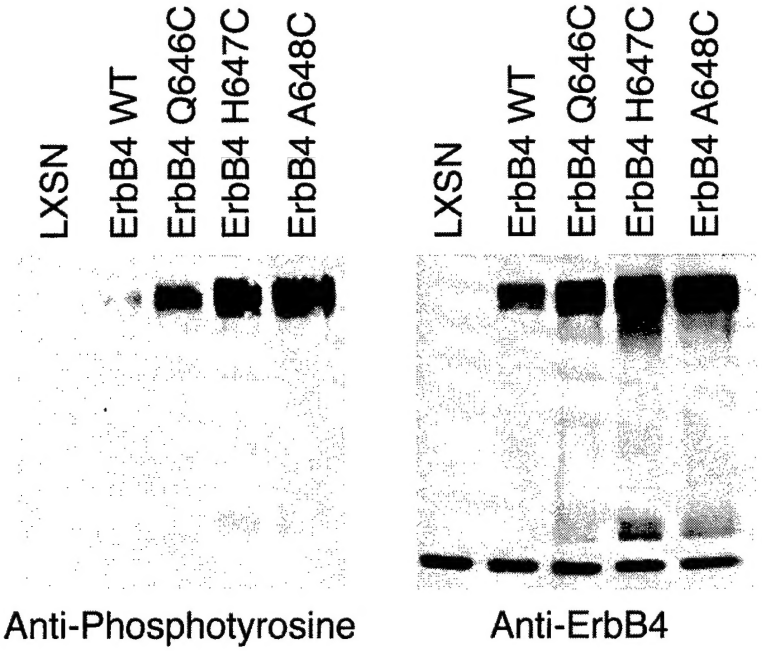

Figure 2. Three ErbB4 mutants exhibit increased kinase activity in an *in vitro* kinase assay.

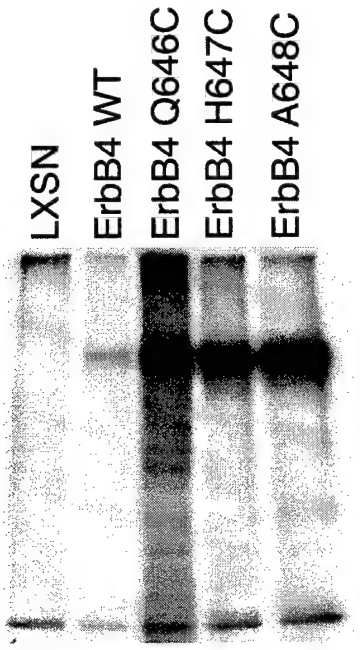

Figure 3. The three constitutively-active ErbB4 mutants do not induce foci in the FR3T3 rodent fibroblast cell line.

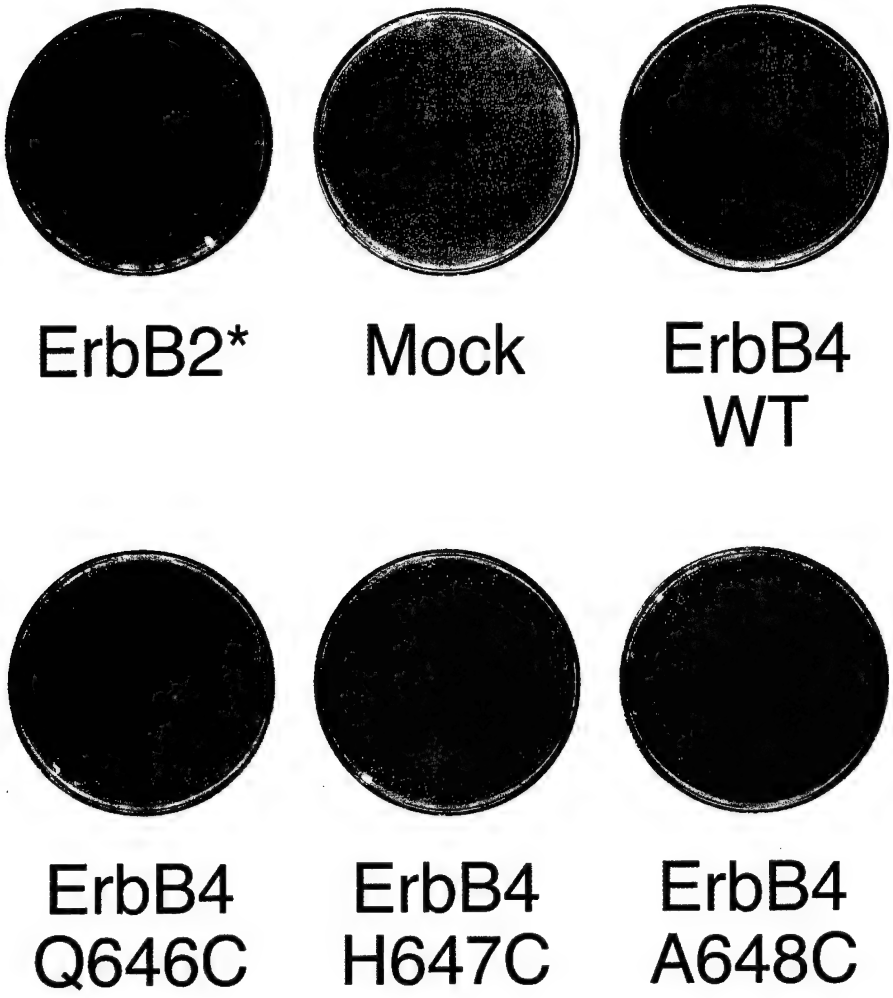

Figure 4. The three constitutively-active ErbB4 mutants do not induce anchorage-independent growth of the FR3T3 rodent fibroblast cell line.

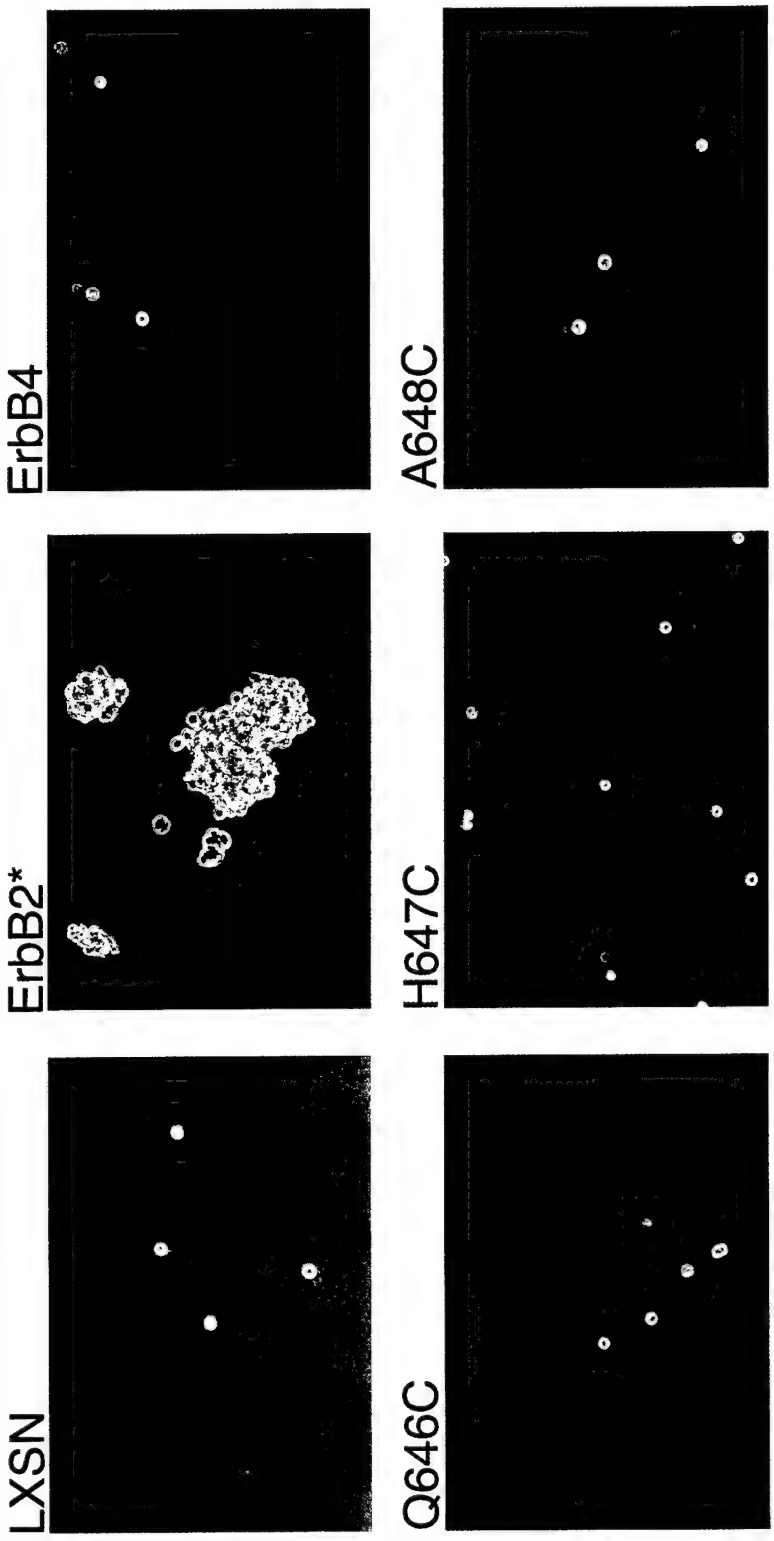

Figure 5. The three constitutively-active ErbB4 mutants do not cause increases in the growth rate or saturation density of the FR3T3 rodent fibroblast cell line.

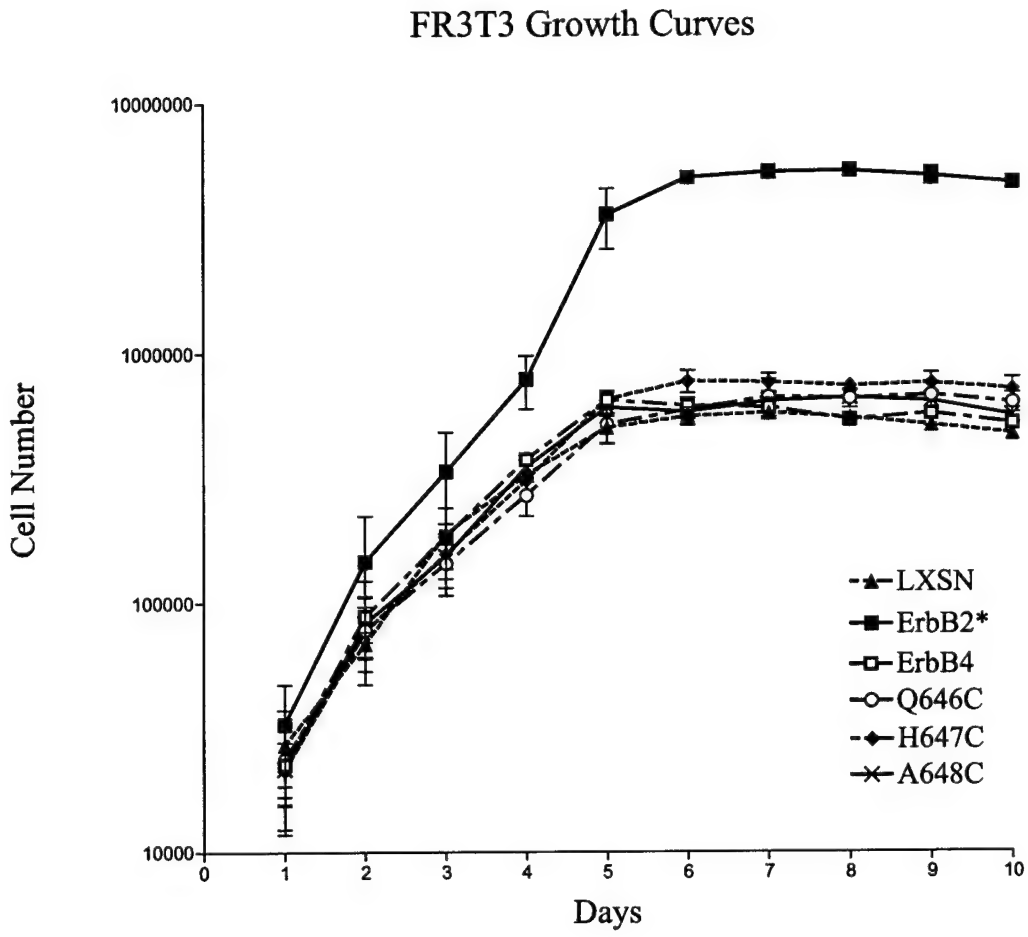

Figure 6. The Q646C constitutively-active ErbB4 mutant is coupled to decreased drug-resistant colony formation in MCF-10A human mammary epithelial cells.

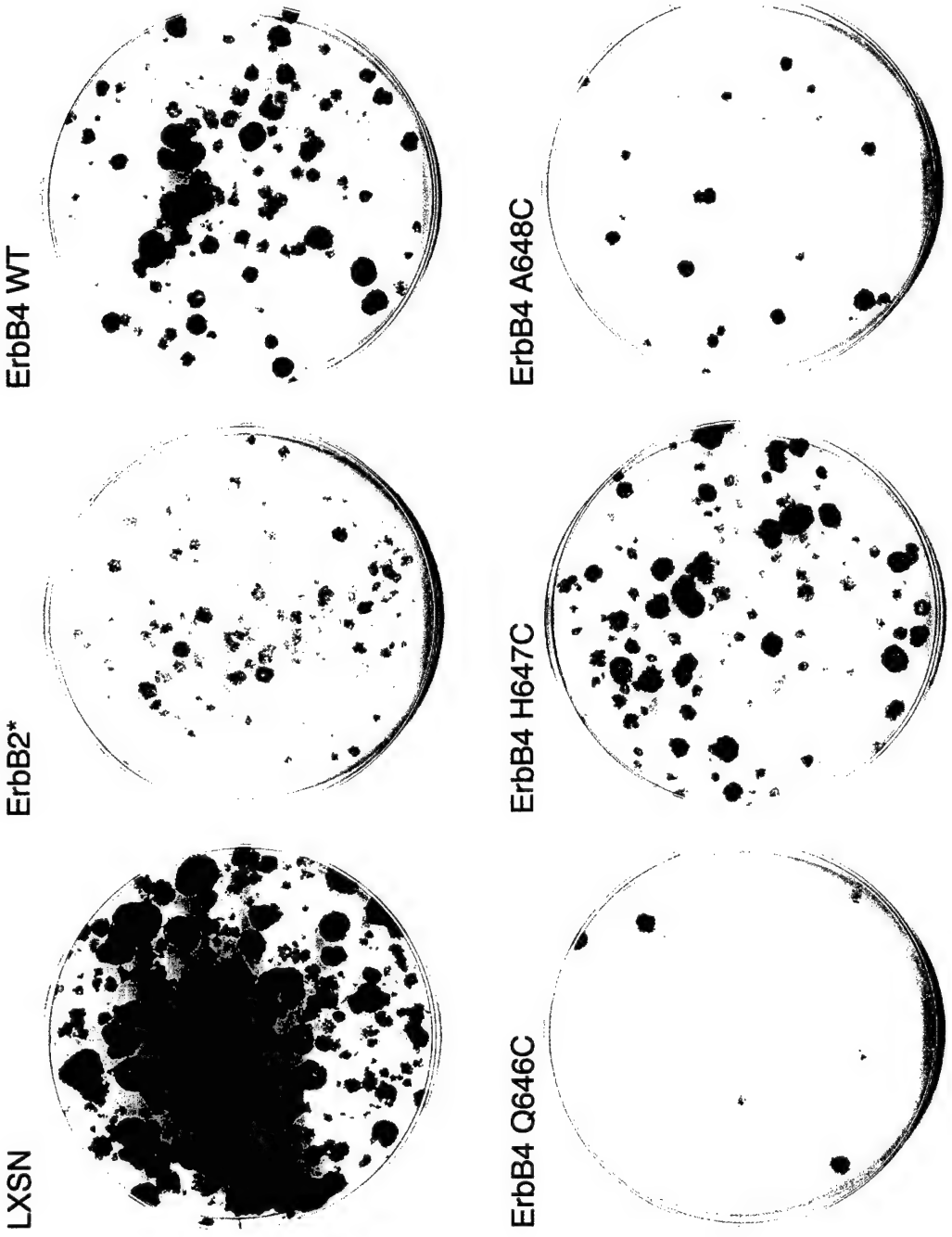

Table 1. The relative titer of the Q646C ErbB4 mutant recombinant retrovirus is much lower in MCF-10A cells than is the titer of the other recombinant retroviruses.

Retrovirus Titers (CFU/ml)			
Retrovirus	**Cell Line**		**Ratio* 1000**
	MCF-10A	**C127**	
Vector	810	258500	3.13
ErbB2*	393	61000	6.44
ErbB4 WT	489	47000	10.39
Q646C ErbB4	**28**	**58000**	0.48
H647C ErbB4	442	72333	6.11
A648C ErbB4	132	23750	5.54

Constitutively-active ErbB4 and ErbB2 Mutants Exhibit Distinct Biological Activities

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Abstract

ErbB4 is a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinases, which includes EGFR/ErbB1, ErbB2/HER2/Neu, and ErbB3/HER3. These receptors play important roles both in normal development and in neoplasia. For example, deregulated signaling by ErbB1 and ErbB2 is observed in many human malignancies. In contrast, the roles that ErbB4 plays in tumorigenesis and normal biological processes have not been clearly defined. To identify the biological responses that are coupled to ErbB4, we have constructed three constitutively-active ErbB4 mutants. Unlike a constitutively-active ErbB2 mutant, the ErbB4 mutants are not coupled to increased cell proliferation, loss of contact inhibition, or anchorage independence in a rodent fibroblast cell line. This suggests that ErbB2 and ErbB4 may play distinct roles in tumorigenesis *in vivo*.

Introduction

ErbB4 (HER4/p180^{erbB4}) is a member of the epidermal growth factor receptor (EGFR/ErbB) family of receptor tyrosine kinases. These receptors play important roles in the embryonic development of heart, lung, and nervous tissues (1-4), and they have been implicated in the progression of metastatic disease. For example, EGFR/ErbB1 is overexpressed, amplified, or mutated in a number of human malignancies including breast, ovary, prostate, and lung cancers (5-7). ErbB2 overexpression correlates with tumor aggressiveness and poor prognosis for survival in node positive breast cancer patients (Reviewed in 8). Furthermore, ErbB3 overexpression is observed in a subset of human mammary and gastric cancers (9, 10).

Some reports indicate that increased ErbB4 expression or signaling is associated with tumorigenesis. ErbB4 overexpression has been observed in a variety of cancers, including tumors of the thyroid, breast, and gastrointestinal tract (11-13). However, the prognostic significance of ErbB4 expression in tumors may also depend on which ErbB family members are co-expressed with ErbB4. In the case of childhood meduloblastoma (one of the most common solid tumors of childhood), patients with tumors overexpressing both ErbB2 and ErbB4 have a significantly worse prognosis for survival than patients with tumors that overexpress either receptor alone (14).

Increased ErbB4 expression or signaling also correlates with tumor cell differentiation and reduced aggressiveness. ErbB4 overexpression in breast tumors is associated with progesterone receptor (PR) and estrogen receptor (ER) expression and a more favorable prognosis (15-17). In contrast, ErbB2 overexpression varies inversely with PR and ER levels and indicates tumors that are more likely to be metastatic and fatal (18). In one survey of common solid human cancers, the loss of ErbB4 expression is seen in a significant percentage of

breast, prostate, and head and neck malignancies (19). These findings raise the intriguing possibility that ErbB4 is unique to the ErbB family of receptors in that ErbB4 expression and signaling may couple to reduced tumorigenesis or tumor cell proliferation. However, in the face of the conflicting evidence we have summarized here it remains unclear what general or specific roles ErbB4 plays in differentiation, tumor suppression, or proliferation.

Efforts to elucidate ErbB4 function have been hampered by many factors. First, there are no known agonists or antagonists specific to the ErbB4 receptor. All of the peptide hormones of the Epidermal Growth Factor (EGF) family that are capable of binding ErbB4 also bind at least one other ErbB family member. For example, epiregulin (EPR) and betacellulin (BTC) bind and activate both ErbB1 and ErbB4 (20, 21). Furthermore, ligands that do not bind an ErbB family receptor can still activate signaling by that receptor in *trans* through ligand-induced receptor heterodimerization (reviewed in 22, 23). For example, EGF stimulates ErbB2 tyrosine phosphorylation when ErbB2 is coexpressed with ErbB1, whereas EGF will not stimulate ErbB2 tyrosine phosphorylation in the absence of ErbB1 (24). Consequently, ligands that bind and directly activate ErbB4 receptor (neuregulin, betacellulin, amphiregulin, and epiregulin) also stimulate ErbB1, ErbB2, and ErbB3 signaling (20, 21, 25, 26, reviewed in 22, 23). Therefore, in most contexts it is virtually impossible to use an EGF family hormone to study the functional consequences of ErbB4 signaling.

To study ErbB4 function we have opted to generate ErbB4 mutants that contain a cysteine substitution in the extracellular domain. This is predicted to result in constitutively-dimerized and constitutively-active ErbB4 mutants. Introducing cysteine residues to form covalently-linked, dimeric, constitutively-active receptor tyrosine kinases is not novel. This strategy has been used to generate dimeric, constitutively-active mutants of EGFR/ErbB1 and

ErbB2 (27,28). Cysteine substitutions also lead to constitutively active mutants of the Fibroblast Growth Factor Receptor 2 (FGFR2) and FGFR3 (29, 30).

Here we report the generation and characterization of three constitutively-active ErbB4 mutants. These mutants were generated through the introduction of a cysteine residue in the extracellular region of ErbB4. These mutants exhibit increased ligand-independent ErbB4 tyrosine phosphorylation, dimerization, and kinase activity. However, these constitutively-active ErbB4 mutants do not induce increased proliferation, loss of contact inhibition, or anchorage-independent growth in Fischer Rat 3T3 fibroblasts. In contrast, a constitutively-active ErbB2 mutant does induce increased proliferation, loss of contact inhibition, and anchorage-independent growth in Fischer Rat 3T3 fibroblasts. These results suggest that ErbB4 and ErbB2 couple to different signaling pathways and biological responses. These results also suggest that ErbB4 and ErbB2 may play distinct roles in tumorigenesis *in vivo*.

Results

ErbB4 mutants are constitutively tyrosine phosphorylated We substituted a single cysteine for amino acids Pro645, Gln646, His647, Ala648 and Arg649 in the juxtamembrane region of the ErbB4 extracellular domain. These ErbB4 mutants (P645C, Q646C, H647C, A648C, and R649C) were generated in the context of the pLXSN-ErbB4 recombinant retroviral expression vector (26). Because these cysteine substitutions might cause inappropriate protein folding and decreased protein stability, we assayed the ErbB4 mutants for stable expression. We transfected the recombinant retroviral vectors containing the ErbB4 mutant constructs into the Ψ2 ecotropic retrovirus packaging cell line, selected for stable transformants, and generated pooled cell lines. We harvested low-titer ecotropic retrovirus stocks from these cell lines and we analyzed the expression and tyrosine phosphorylation of the ErbB4 mutants in these cell lines. Three ErbB4 mutants (Q646C, H647C, and A648C) exhibit abundant expression and ligand-independent tyrosine phosphorylation (data not shown). However, the R649C ErbB4 mutant is not efficiently expressed and the P645C mutant does not display ligand-independent tyrosine phosphorylation (data not shown).

Overexpression of ErbB family receptors causes ligand-independent receptor tyrosine phosphorylation (31-33). Consequently, we were concerned that the ligand-independent phosphorylation of the Q646C, H647C, and A648C ErbB4 mutants in the transfected Ψ2 cells was a consequence of overexpression. Therefore, we infected the PA317 amphotropic retrovirus packaging cell line with the ErbB4 mutant recombinant ecotropic retroviruses at low multiplicities of infection (MOI less than 0.1), selected for infected cells, and generated pooled cell lines. Since these cell lines were generated by infection at low multiplicities of infection, it

is likely that each cell contains only one or two copies of the ErbB4 expression construct. This reduces the likelihood of ErbB4 overexpression in these cell lines.

We analyzed ErbB4 expression and tyrosine phosphorylation in the PA317 cell lines by anti-ErbB4 immunoprecipitation and either anti-ErbB4 (Figure 1, right panel) or anti-phosphotyrosine (Figure 1, left panel) immunoblotting. As expected, cells infected with the LXSN vector control retrovirus do not exhibit ErbB4 expression (Figure 1, right panel) or tyrosine phosphorylation (Figure 1, left panel). Cells infected with the wild-type or mutant ErbB4 retroviruses exhibit ErbB4 expression (Figure 1, right panel). However, cells infected with the mutant ErbB4 retroviruses exhibit abundant ErbB4 tyrosine phosphorylation, whereas cells infected with the wild-type ErbB4 retrovirus exhibit minimal ErbB4 tyrosine phosphorylation (Figure 1, left panel).

Quantification of the chemilumigrams shown in Figure 1 suggests that the expression levels of the three ErbB4 mutants is less than three times greater than the amount of wild-type ErbB4 expression (Table 1). In contrast, the amounts of tyrosine phosphorylation of the three ErbB4 mutants appear to be much greater than the amount of wild-type ErbB4 tyrosine phosphorylation. Moreover, the ratios of ErbB4 tyrosine phosphorylation to ErbB4 expression for the three ErbB4 mutants appear to be at least four times greater than the ratio for wild-type ErbB4. These data suggest that the three ErbB4 mutants exhibit greater amounts of tyrosine phosphorylation on a per molecule basis than does wild-type ErbB4. Consequently, these data indicate that the Q646C, H647C, and A648C ErbB4 mutants are constitutively active for signaling.

ErbB4 mutants have increased *in vitro* kinase activity. Next, we assessed whether the increased tyrosine phosphorylation of the three ErbB4 mutants correlates with increased kinase

activity. Lysates were prepared from PA317 cells that express wild-type ErbB4 or the ErbB4 mutants. Equal amounts of lysate were immunoprecipitated with an anti-ErbB4 polyclonal antibody. Kinase reactions were performed on the immunoprecipitates in the presence of [γ-^{32}P] ATP. The reaction products were resolved by SDS/PAGE on a 7.5% acrylamide gel. The gel was dried and the reaction products were visualized by autoradiography.

In Figure 2, we show that PA317 cells infected with the LXSN vector control retrovirus lack detectable ErbB4 kinase activity. Moreover, PA317 cells that express the three constitutively-active ErbB4 mutants exhibit greater ErbB4 tyrosine kinase activity than cells that express wild-type ErbB4. Quantification of the bands on the autoradiogram indicates that the Q646C and H647C ErbB4 mutants exhibit approximately 5-fold more kinase activity than does wild-type ErbB4, whereas the A648C ErbB4 mutant exhibits approximately 9-fold more kinase activity than does wild type ErbB4. Given that the expression of the ErbB4 mutants is somewhat greater than the expression of wild-type ErbB4 (Table 1), it appears that the intrinsic kinase activity of the three ErbB4 mutants is approximately 2-fold greater than the intrinsic kinase activity of wild-type ErbB4.

Constitutively-active ErbB4 mutants do not induce a loss of contact inhibition. Once we determined that the Q646C, H647C, and A648C ErbB4 mutants are constitutively active for signaling, we performed experiments using these mutants to identify the biological events coupled to ErbB4 signaling. A common assay for genes that encode growth control or signaling proteins involves transfecting an established rodent fibroblast cell line with the gene and assaying the transfected cells for foci of piled-up cells. These foci indicate a loss of contact inhibition, a common attribute of malignant cells. Thus, this transfection assay is commonly used to identify genes that encode proteins that are coupled to malignant growth transformation.

Conflicting results have been obtained from assays for growth transformation by ErbB4. Transfection and consequent overexpression of ErbB4 induces foci (loss of contact inhibition) in NIH 3T3 clone 7 cells in the absence of ligand. Moreover, in these cells focus formation was stimulated by the ErbB4 ligand Neuregulin 2β (31). In contrast, NIH 3T3 clone 7d cells (which lack EGFR expression) transfected with wild-type ErbB4 did not form foci in the presence or absence of neuregulin 1β; however, ErbB4 cotransfected with EGFR/ErbB1 or ErbB2 does induce foci in these cells (32,33). One possible explanation is that ErbB4 lacks intrinsic transforming activity, but does permit EGFR/ErbB1 or ErbB2 signaling and coupling to growth transformation in the presence of an ErbB4 ligand.

To test whether ErbB4 signaling is sufficient to transform the growth of cultured rodent fibroblasts, Fischer Rat 3T3 (FR3T3) fibroblasts were infected with 200 Cfu of the ErbB4 mutant recombinant ecotropic retrovirus stocks and assayed for focus formation. Cells infected with the LXSN vector control recombinant ecotropic retrovirus and with the wild-type ErbB4 recombinant ecotropic retrovirus served as negative controls. Cells infected with the constitutively-active mutant ErbB2* retrovirus served as a positive control.

FR3T3 cells infected with the ErbB2* retrovirus formed foci nine days after infection, whereas cells infected with the vector control retrovirus did not (Figure 3). In contrast, cells infected with the wild-type or mutant ErbB4 retroviruses did not form foci. Eighteen days after infection, the foci arising from FR3T3 cells infected with the ErbB2* retrovirus completely covered the surface of the tissue culture plate and had begun to detach from the surface of the plate (data not shown). In contrast, the FR3T3 cells infected with the vector control retrovirus did not form any foci. FR3T3 cells infected with the constitutively-active ErbB4 mutant retroviruses formed a few foci; however, the number of foci was comparable to the number seen

in FR3T3 cells infected with the wild-type ErbB4 retrovirus. Thus, while the constitutively-active ErbB2* mutant readily induces foci in FR3T3 fibroblasts, the constitutively-active ErbB4 mutants do not. This suggests that ErbB2 and ErbB4 are coupled to distinct cellular signaling pathways and biological events.

Constitutively-active ErbB4 mutants do not induce anchorage-independent growth. Next we assayed FR3T3 cells that express the constitutively-active ErbB4 mutants for growth while suspended in semi-solid medium. Because anchorage-independent growth is another characteristic attribute of tumor cells *in vivo*, this assay is another way to determine whether ErbB4 signaling is coupled to malignant growth transformation.

FR3T3 cells were infected with the ErbB4 mutant recombinant ecotropic retroviruses at a low multiplicity of infection and infected cells were selected using G418. Drug-resistant colonies of cells were pooled and expanded into cell lines. Control cell lines were generated through infection of FR3T3 cells with the wild-type ErbB4 retrovirus, the constitutively-active ErbB2 retrovirus, and with the LXSN vector control retrovirus. These cell lines were seeded at a density of $2x10^4$ cells/ml in 60 mm dishes in semi-solid medium containing 0.3% low melting point (LMP) agarose. Fresh medium containing LMP-agarose was added every three days. Photographs were taken of representative fields after ten days.

FR3T3 cells that express the constitutively-active ErbB2* mutant exhibit anchorage-independent growth (Figure 4). In contrast, cells that were infected with the LXSN recombinant retroviral vector control and cells that express wild-type ErbB4 or the ErbB4 mutants do not exhibit anchorage-independent growth. The results of this assay are consistent with the results of the focus formation assay; both assays indicate that ErbB4 signaling is distinct from ErbB2

signaling in that ErbB4 signaling is not coupled to malignant growth transformation in FR3T3 fibroblasts.

Constitutively-active ErbB4 mutants do not increase the growth rate or saturation density. Another characteristic of malignantly-transformed fibroblasts is that their growth rates and saturation densities are higher than those of their nontransformed counterparts. Indeed, constitutive ErbB2 signaling is coupled to increased growth rates (Reviewed in 8). Thus, we assessed whether the constitutively-active ErbB4 mutants affected the growth rate or saturation density of FR3T3 fibroblasts. The FR3T3 cell lines described earlier were seeded in 60 mm dishes at a density of 2×10^4 cells per dish (700 cells/cm^2). Cells were incubated for 10 days to permit proliferation. During this period cells were counted every twenty-four hours.

The growth rate of the cells that express ErbB2* is slightly greater than the growth rates of the other cell lines (Figure 5). Note that the growth rates of the cells that express the constitutively-active ErbB4 mutants are indistinguishable from the growth rates of cell lines that express wild-type ErbB4 or the vector control. The growth curves in Figure 5 were used to determine the saturation densities for the six cell lines (Table 2). Note that the saturation density of the cell line that expresses ErbB2* is higher than the saturation densities of the other cell lines. Moreover, the saturation densities of the cell lines that express the ErbB4 mutants are not markedly higher than the saturation densities of the vector control cell line or the cell line that expresses wild-type ErbB4. Once again, these data suggest that constitutive ErbB4 signaling is not coupled to malignant growth transformation in fibroblasts. Thus, the signaling pathways and biological responses that are coupled to ErbB4 are distinct from those that are coupled to ErbB2.

Discussion

In this report we describe the construction and initial characterization of three constitutively-active ErbB4 mutants. These mutants display increased dimerization (data not shown), ligand-independent tyrosine phosphorylation and kinase activity. In these respects, the ErbB4 mutants resemble constitutively-active mutants of ErbB2 or EGFR. However, unlike constitutively-active ErbB2 mutants, these mutants are not coupled to malignant growth transformation in FR3T3 fibroblasts; they do not induce foci, anchorage-independent growth, or increases in the growth rate or saturation density. These data suggest that ErbB2 and ErbB4 play distinct roles in tumorigenesis *in vivo*.

Clearly, additional work is necessary to define the roles that ErbB4 plays in tumorigenesis and in regulating cellular functions *in vivo*. However, important clues have emerged to guide these future studies. In a significant percentage of breast and prostate tumor samples ErbB4 expression is reduced or lost, and tumor aggressiveness appears to inversely correlate with ErbB4 expression (15-17). Moreover, ligands for ErbB4 can induce terminal differentiation and growth arrest of some mammary tumor cell lines (34, 35). These data indicate that ErbB4 signaling may be coupled to differentiation, growth arrest, and tumor suppression. The ErbB4 mutants described in this study will enable us to evaluate this hypothesis. Indeed, preliminary data from our laboratory indicate that the Q646C ErbB4 mutant causes reduced colony formation in plastic dishes by a number of cultured human breast and prostate tumor cell lines.

We will also perform additional studies to characterize the biochemistry of signaling by the three ErbB4 mutants. Whereas these mutants exhibit greater ligand-independent tyrosine phosphorylation and autokinase activity than the wild-type receptor, it is unclear whether this is

due to increased intrinsic kinase activity or due to increased availability of substrate. Additional experiments are warranted to distinguish between these two possibilities.

Another area of future study will focus on identifying the mechanisms by which ErbB4 is coupled to biological responses. Initial studies will identify the sites of ErbB4 tyrosine phosphorylation for these mutants. If our preliminary studies indicating that the Q646C ErbB4 mutant is coupled to prostate and mammary tumor cell growth arrest hold true, then we will use genetic strategies to identify the sites of ErbB4 tyrosine phosphorylation that are sufficient and necessary to couple the Q646C ErbB4 mutant to this biological response. A similar strategy has been used to identify the sites of ErbB2 and platelet-derived growth factor receptor tyrosine phosphorylation that are critical for coupling these receptors to biological responses (36, 37).

Once we have identified the site(s) of tyrosine phosphorylation that is sufficient for coupling to biological responses, we will identify signaling proteins that bind this phosphorylation site and couple it to biological responses. Using this strategy we will begin to construct the ErbB4 signaling pathway. Our prediction is that the three constitutively-active ErbB4 mutants are phosphorylated on different tyrosine residues and that these mutants differentially couple to biological responses. We have previously shown that different ErbB4 ligands cause phosphorylation on different sites on ErbB4 and differential coupling to biological responses (38). Moreover, one cysteine substitution mutation in the rat ErbB2 extracellular domain (V656C) results in low amounts of constitutive receptor tyrosine phosphorylation and efficient coupling to malignant growth transformation in rodent fibroblasts. In contrast, another rat ErbB2 extracellular domain cysteine substitution mutant (T657C) exhibits very high levels of constitutive receptor tyrosine phosphorylation but a relatively low amount of coupling to malignant growth transformation in rodent fibroblasts (28).

We were somewhat surprised to discover that the three constitutively-active ErbB4 mutants failed to coupled to malignant growth transformation in a rodent fibroblasts cell line. Nonetheless, these mutants will enable us to assess ErbB4 function in a wide variety of cell, tissue, and organismal contexts. Given that ErbB4 appears to regulate diverse functions in a number of distinct contexts, much work remains to complete this story.

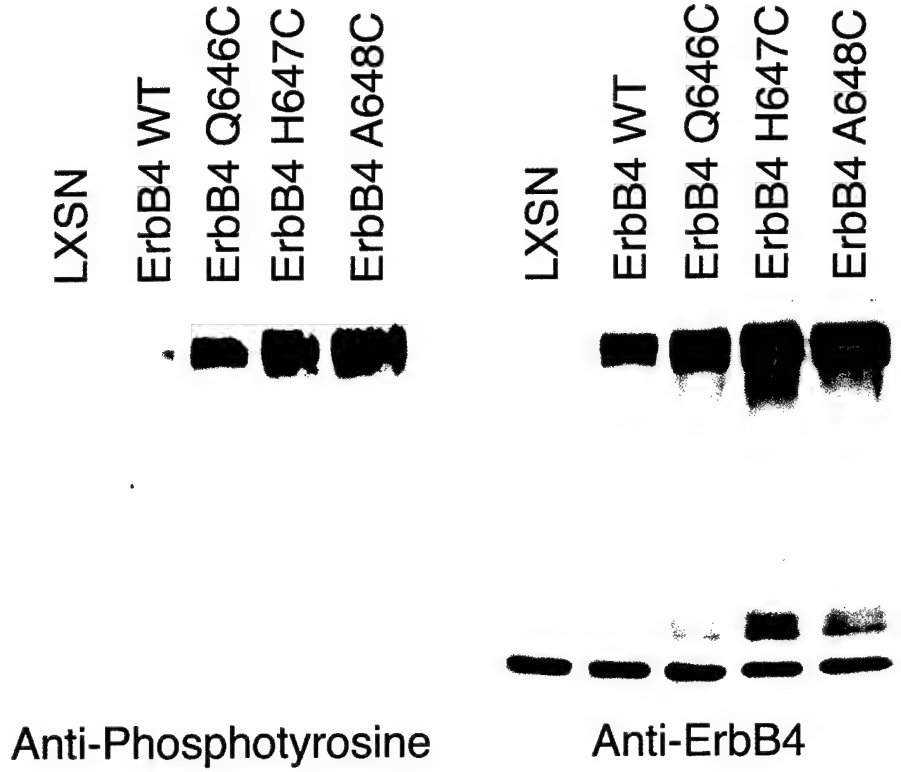

Figure 1. ErbB4 mutants are constitutively tyrosine phosphorylated. ErbB4 expression and tyrosine phosphorylation were assayed in PA317 cells infected with retroviruses that direct the expression of wild-type ErbB4 or the ErbB4 mutants. Cells infected with the LXSN recombinant retrovirus vector control served as the negative control. Lysates were prepared from each of the cell lines and ErbB4 was immunoprecipitated from 1000 μg of each lysate. Samples were resolved by SDS-PAGE, electroblotted to nitrocellulose, and immunoblotted with an anti-phosphotyrosine antibody (left panel). The blot was then stripped and probed with an anti-ErbB4 rabbit polyclonal antibody (right panel). ErbB4 is represented by the band at the top of the blots.

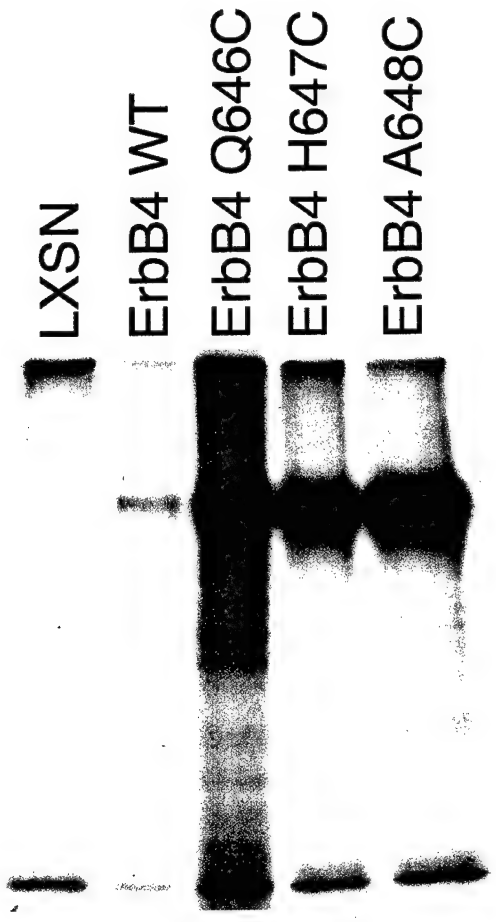

Figure 2. Q646C, H647C, and A648C mutants exhibit increased *in vitro* kinase activity. Equal amounts of protein lysates from PA317 cells that stably express wild-type ErbB4 or the ErbB4 mutants (Q646C, H647C, A648C) were immunoprecipitated with an anti-ErbB4 rabbit polyclonal antibody. Lysates from PA317 cells that express the LXSN vector served as the negative control. Kinase reactions were performed on the immunoprecipitates in the presence of [γ-^{32}P]ATP. The products were resolved by SDS-PAGE. The gel was dried overnight and exposed to X-ray film for approximately 20 hours to visualize the products of the kinase reactions.

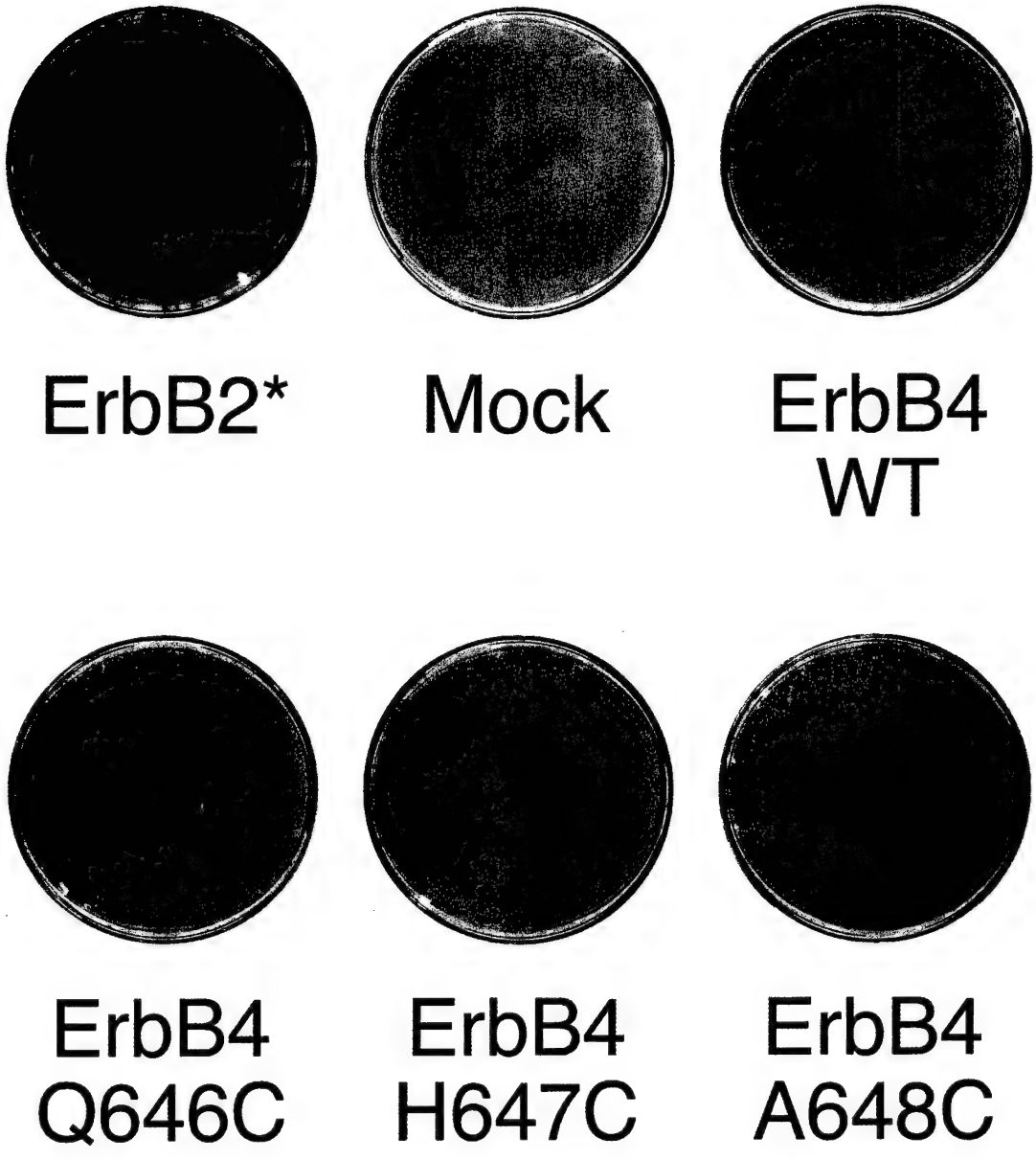

Figure 3. Constitutively-active ErbB4 receptors do not induce a loss of contact inhibition. FR3T3 fibroblasts infected with the LXSN (vector control) retrovirus, the wild-type ErbB4 retrovirus, the constitutively-active ErbB2* retrovirus, or the constitutively-active ErbB4 mutant retroviruses were assayed for loss of contact inhibition (focus formation).

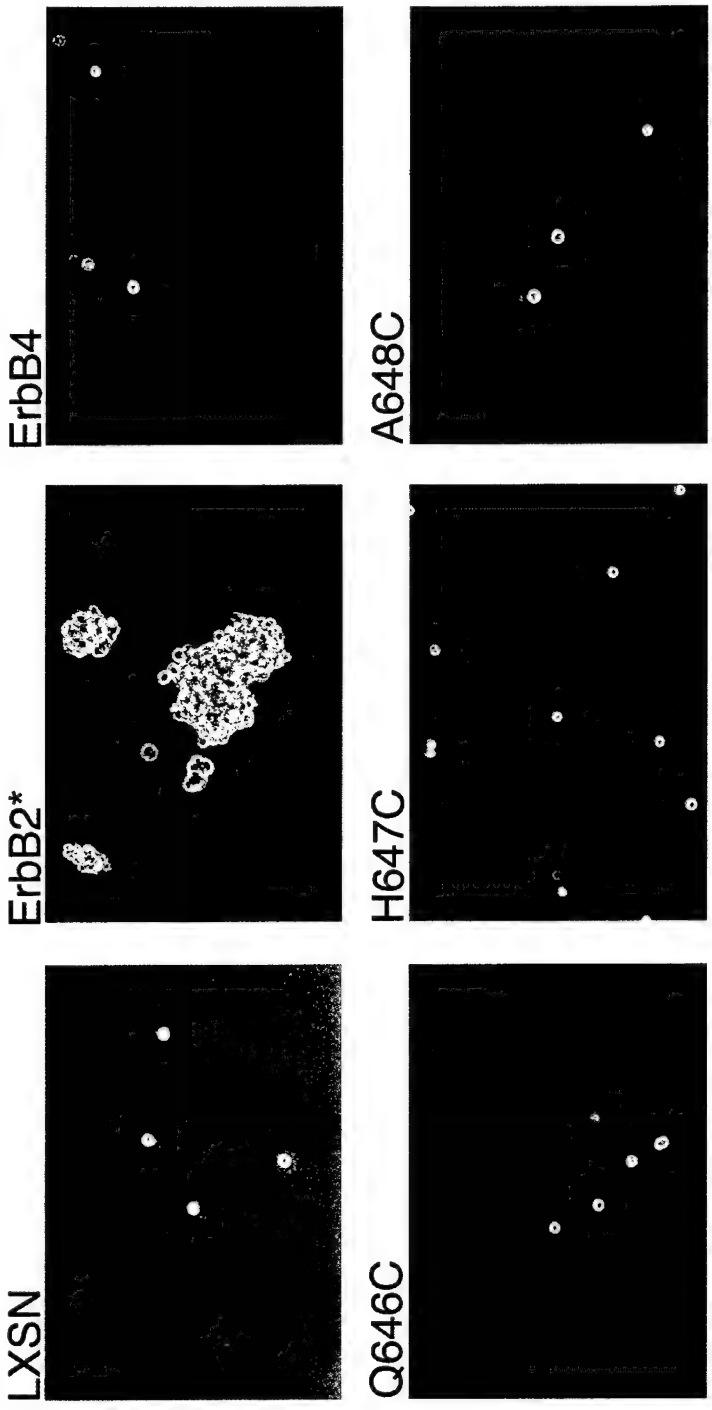

Figure 4. Constitutively-active ErbB4 receptors do not induce growth in semi-solid medium. FR3T3 cells that stably express the LXSN vector control, the constitutively-active ErbB2 mutant (ErbB2*), wild-type ErbB4, or the constitutively-active ErbB4 mutants (Q646C, H647C, A648C) were seeded in semi-solid medium at a density of 2 x 10^4 cells/ml in 60 mm dishes. The cells were incubated for 10 days, after which images were recorded by photomicroscopy. Images shown are representative of those obtained in three independent experiments.

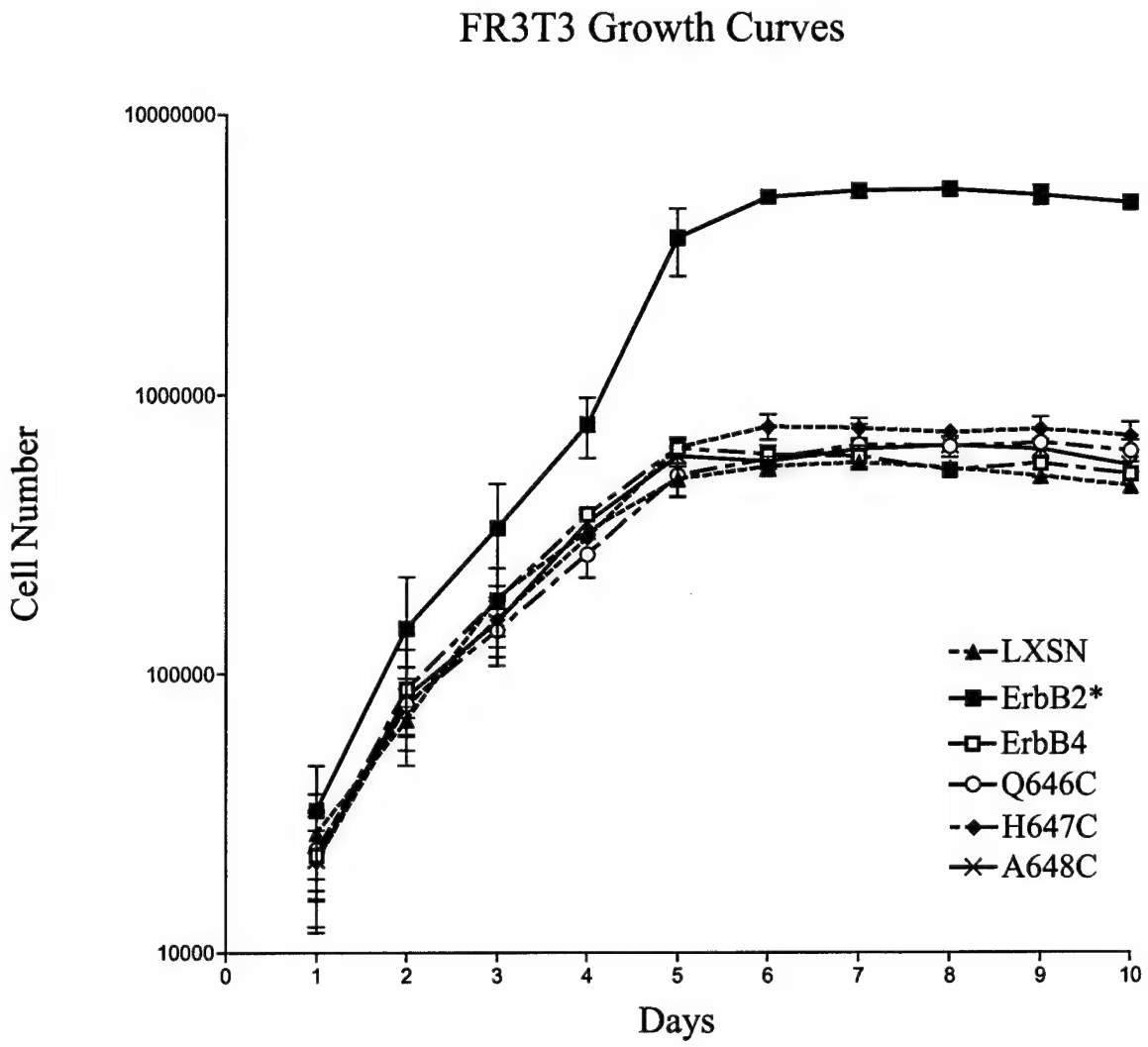

Figure 5. Constitutively-active ErbB4 mutants do not increase the growth rate of FR3T3 fibroblasts. FR3T3 cells that express the LXSN vector control, the constitutively-active ErbB2* mutant, wild-type ErbB4, or the constitutively-active ErbB4 mutants (Q646C, H647C, A648C) were plated at a density of 2 x 10^4 cells in 60 mm dishes (700 cells/cm^2) and were incubated for one to ten days. Cells were counted daily to assess growth rates and saturation densities. The means for three independent experiments are shown with error bars that represent the standard errors of the means.

Table 1. The Q646C, H647C, and A648C ErbB4 mutants exhibit increased normalized tyrosine phosphorylation.

Cell Line	ErbB4 Tyrosine Phosphorylation	ErbB4 Expression	Ratio
Wild-type ErbB4	212867	1831340	0.116
ErbB4 Q646C	1860472	3329432	0.558
ErbB4 H647C	2944443	4659376	0.631
ErbB4 A648C	3981658	4509147	0.883

Table 2. Constitutively-active ErbB4 mutants do not increase the saturation density of FR3T3 fibroblasts.

	Saturation Densities
LXSN	$5.8 \pm 0.3 \times 10^5$
ErbB2*	$5.4 \pm 0.1 \times 10^6$
ErbB4	$6.1 \pm 0.5 \times 10^5$
Q646C	$6.6 \pm 0.6 \times 10^5$
H647C	$7.6 \pm 0.7 \times 10^5$
A648C	$6.6 \pm 0.4 \times 10^5$

Materials and Methods

Cell Lines, Cell Culture, and Antibodies. The Ψ2, PA317, and FR3T3 cell lines were generous gifts from Daniel DiMaio, Yale University. All cell lines were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 IU/ml penicillin, 50 μg/ml streptomycin (Mediatech), and 0.25 μg/ml fungizone (Amphotericin B; Gibco/BRL). Recombinant cell lines generated in the course of the following experiments were propagated in the media described above supplemented with 200 μg/ml G418 (Mediatech).

The anti-ErbB4 mouse monoclonal (SC-8050), anti-ErbB4 rabbit polyclonal (SC-283), and anti-ErbB2 rabbit polyclonal (C-18) antibodies were purchased from Santa Cruz Biotechnology. Goat anti-mouse and goat anti-rabbit horseradish peroxidase conjugated antibodies were purchased from Pierce. Enhanced chemiluminescence (ECL) Western blotting reagents, Redivue™ adenosine 5'-[γ-^{32}P] –triphosphate, and Protein-A Sepharose (CL-4B) were purchased from Amersham Pharmacia Biotech. The 4G10 anti-phosphotyrosine mouse monoclonal antibody was purchased from Upstate Biotechnology.

Plasmids. The recombinant retroviral vector pLXSN (39) was obtained from Daniel DiMaio, Yale University. This construct contains two recombinant LTRs derived from the Maloney Murine Leukemia Virus and the Maloney Murine Sarcoma Virus. These LTRs flank the Ψ packaging signal and the aminoglycoside 3'-phosphotransferase (Neo^R) gene under the transcriptional control of the SV40 early promoter. The Neo^R gene confers resistance to the aminoglycoside antibiotic G418 (geneticin, Gibco/BRL).

The recombinant retroviral construct pLXSN-ErbB4 (26) was generated by subcloning the human ErbB4 cDNA into pLXSN. In this construct the ErbB4 cDNA is under the transcriptional control of the upstream LTR. The recombinant retroviral construct pLXSN-ErbB2* (40) was a gift of Lisa Petti, Albany Medical College. It was generated by subcloning the cDNA encoding the constitutively active rat ErbB2 mutant (ErbB2*) into pLXSN. In this construct the ErbB2* cDNA is under the transcriptional control of the upstream LTR.

ErbB4 Mutagenesis. The plasmid pLXSN-ErbB4 was used as the template for site-directed mutagenesis (QuikChange™ Site Directed Mutagenesis Kit, Stratagene) to construct the putative constitutively-active ErbB4 mutants. The mutants were constructed by introducing mutations that substitute a cysteine residue for Proline645, Glutamine646, Histidine647, Alanine648, or Arginine649 in the ErbB4 extracellular juxtamembrane domain. These mutants are denoted as follows: P645C, Q646C, H647C, A648C, and R649C. A new restriction enzyme site was also engineered in each mutant to facilitate the identification of the mutants. The following primers were used for mutagenesis. "T" denotes the upper primer, while "B" denotes the lower primer. The novel cysteine codons and anticodons are indicated by bold type, the point mutations that create the novel cysteine residues are double underlined, and the novel restriction enzyme sites are singly underlined.

P645CT: 5'-ATTTACTACCCATGGACCGGTCATTCCACTTTA**TGC**CAACATGCTAGAACTCCC-3'

P645CB: 5'-GGGAGTTCTAGCATGTTG**GCA**TAAAGTGGAATGACCGGTCCATGGGTAGTAAAT-3'

Q646CT: 5'-TACTACCCATGGACCGGTCATTCCACTTTACCA**TGC**CATGCTAGAACTCCCCTG-3'

Q646CB: 5'-CAGGGGAGTTCTAGCATG**GCA**TGGTAAAGTGGAATGACCGGTCCATGGGTAGTA-3'

H647CT: 5'-CATTTACTACCCATGGACCGGTCATTCCACTTTACCACAA**TGT**GCTAGAACTCCCCT-3'

H647CB: 5'-AGGGGAGTTCTAGC**ACA**TTGTGGTAAAGTGGAATGACCGGTCCATGGGTAGTAAATG-3'

A648CT: 5'-TCCACTTTACCACAACATTGTAGAACTCCTCTGATTGCAGCTGGA-3'

A648CB: 5'-TCCAGCTGCAATCAGAGGAGTTCTACAATGTTGTGGTAAAGTGGA-3'

R649CT: 5'-ACTTTACCACAACATGCTTGCACTCCTCTGATTGCAGCTGGA-3'

R649CB: 5'-TCCAGCTGCAATCAGAGGAGTGCAAGCATGTTGTGGTAAAGT-3'

The site-directed mutagenesis reactions were performed according to manufacturer's instructions. Standard techniques (41) were used for bacterial transformations, small-scale plasmid DNA preparations, restriction enzyme analysis of the clones, and large-scale plasmid DNA preparations. Positive clones were sequenced by the University of Wisconsin Biotechnology Center to confirm their identity.

Production of Recombinant Retroviruses and Retroviral Infections. The ErbB4 mutant constructs were transfected using standard techniques (42, 43) into the ψ2 ecotropic retrovirus packaging cell line (44) to generate cell lines that express the ErbB4 mutants and to package the constructs into low-titer ecotropic retrovirus particles (42, 43). ψ2 cells were transfected with the pLXSN vector control plasmid, pLXSN-ErbB4, and pLXSN-ErbB2* to generate control cell lines and recombinant ecotropic retroviruses. The PA317 amphotropic packaging cell line (45) and the FR3T3 rat fibroblast cell line were infected with the ecotropic recombinant retroviruses using standard techniques (42, 43) to generate additional cell lines that express the ErbB4 mutants.

Immunoblot Assays for Receptor Tyrosine Phosphorylation and Expression. The analysis of ErbB4 and ErbB2 tyrosine phosphorylation by immunoprecipitation and anti-phosphotyrosine immunoblotting has been described previously (21, 26). Briefly, cell lysates were generated and protein content was quantified using a Coomassie® Protein Assay Reagent

(46; Pierce Chemical). ErbB2 or ErbB4 was immunoprecipitated from equal amounts of protein using specific antibodies. The immunoprecipitates were resolved by SDS/PAGE on a 7.5% acrylamide gel and electrotransferred onto nitrocellulose. The blots were probed with the anti-phosphotyrosine monoclonal antibody 4G10. Antibody binding was detected and visualized using a goat anti-mouse horseradish peroxidase coupled antibody and enhanced chemiluminescence. The blots were then stripped and probed with the anti-ErbB4 polyclonal antibody to assess ErbB4 expression levels. Antibody binding was detected and visualized using a goat anti-mouse horseradish peroxidase coupled antibody and enhanced chemiluminescence.

The amounts of receptor tyrosine phosphorylation and expression were quantified by digitizing the chemilumigrams using a Linotype-Hell Jade 2-dimensional scanning densitometer set at 600 dpi resolution. The bands on the images were quantified using NIH Image for Macintosh v1.6 software. Values are expressed as arbitrary units. Background levels were computed using the vector control lanes and were subtracted from the gross values to produce net receptor expression and tyrosine phosphorylation values. The digitized images were also cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software.

***In Vitro* Kinase Assay.** ErbB2 and ErbB4 were immunoprecipitated from protein extracts from PA317 cells as previously described (26). Immune complex kinase reactions were performed as previously described (31). Briefly, 35 µl Protein-A Sepharose and 5 µl of anti-ErbB2 or anti-ErbB4 rabbit polyclonal antibodies were used to immunoprecipitate the receptors from lysates containing the same amount of protein. Immunoprecipitates were washed five times in 500 µl kinase buffer (20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 3 mM $MnCl_2$). After the last wash, the samples were resuspended in 100 µl kinase buffer supplemented with 10 µCi of [γ-^{32}P]ATP and were incubated for 10 minutes at room temperature to permit the kinase reaction to

occur. The beads were then washed two times in NET-N (47) and boiled for 5 minutes in SDS/PAGE protein sample buffer. The samples were resolved by SDS/PAGE on a 7.5% acrylamide gel. The gels were dried overnight and exposed to X-ray film for approximately 20 hours. The autoradiograms were digitized using a Linotype-Hell Jade 2-dimensional scanning densitometer set at 600 dpi resolution. The bands on the images were quantified using NIH Image for Macintosh v1.6 software. Values are expressed as arbitrary units. Background levels were computed using the vector control lanes and were subtracted from the gross values to produce net kinase activity values. The digitized images were also cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software.

Focus Formation Assay for Loss of Contact Inhibition. FR3T3 cells were infected with recombinant ecotropic retroviruses as described earlier and in previously-published reports (42, 43). Briefly, 60 mm dishes of cells at approximately 70% confluence were infected with ecotropic retrovirus stocks. Approximately 24 hours after infection, cells were passaged into three 60 mm dishes. Cells were maintained in DMEM supplemented with 10% FBS until foci appeared. The medium was changed every three days. Cells were fixed in 100% methanol and stained with Giemsa (Fisher) to visualize the foci. The plates were digitized using a Linotype-Hell Jade 2-dimensional scanning densitometer set at 600 dpi resolution. The digitized images were cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software.

Assay for Anchorage Independence. FR3T3 cells were seeded at a density of 2×10^4 cells in 60 mm dishes containing 2.5 ml of 0.3% Low Melting Point Agarose (LMP-agarose; Gibco/BRL) as described previously (48). Every three days DMEM supplemented with 10% FBS and 0.3% LMP-agarose was added to each plated. The cells were incubated at 37°C for 10 days and fields were photographed with an Olympus OM-10 camera attached to an Olympus

CK-2 phase-contrast inverted microscope. The images were digitized by the photofinishing firm. These images were cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software. Images are representative of three independent experiments.

Growth Rate/Saturation Density Assay. Stable FR3T3 cell lines expressing the wild-type ErbB4 receptor, ErbB2*, or the ErbB4 mutants (Q646C, H647C, A648C) were plated in ten 60 mm dishes at a density of 2×10^4 cells per dish. Cells were incubated from one to ten days at 37°C. Cells were counted (Coulter Counter® ZM) each day for a total of ten days. The mean and standard error of the mean (SEM) are representative of three independent experiments.

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CONSTRUCTION AND ANALYSIS OF CONSTITUTIVELY-ACTIVE MUTANTS OF THE ERBB4 RECEPTOR TYROSINE KINASE

A Thesis

Submitted to the Faculty

Of

Purdue University

By

Desi Jay Penington

In Partial Fulfillment of the

Requirements for the Degree

Of

Master of Science

August 2001

Dedicated to my parents

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Mom and Dad this is for you.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AR	Amphiregulin
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
BTC	Betacellulin
Cfu	Colony forming units
DMEM	Dulbecco's Modified Eagle's Medium
ECL	Enhanced Chemiluminescence
EDTA	Ethylene Diamine Tetra Acetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EPR	Epiregulin
ER	Estrogen Receptor
FBS	Fetal Bovine Serum
FGFR	Fibroblast Growth Factor Receptor
HB-EGF	Heparin-binding EGF-like Growth Factor
HRP	Horseradish Peroxidase
Ig3	Immumoglobulin-Like Domain 3
LMP	Low Melting Point Agarose
MAPK	Mitogen-Activated Protein Kinase
Neo^R	Neomycin-Resistance Gene
NRG	Neuregulin
PAGE	Polyacrylamide Gel Electrophoresis
PI3K	Phosphatidylinositol 3'-Kinase
PKC	Protein Kinase C

PMA	Phorbol 12-Myristate 13-Acetate
PR	Progesterone Receptor
PTB	Phosphotyrosine Binding Domain
RTK	Receptor Tyrosine Kinase
SDS	Sodium Dodecyl Sulfate
SH2	Src Homology Domain
TBS-T	Tris Buffered Normal Saline supplemented with Tween-20
TDI	Thanatophoric Dysplasia Type I
TGF-α	Transforming Growth Factor-alpha

ABSTRACT

Penington, Desi J., M.S., Purdue University, August 2001. Construction and Analysis of Constitutively-Active Mutants of the ErbB4 Receptor Tyrosine Kinase. Major Professor: Dr. David J. Riese II

ErbB4 is a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinases, which includes ErbB1/EGFR, ErbB2/HER2/Neu, and ErbB3/HER3. These receptors play important roles in both normal development and neoplasia. Previous studies have demonstrated that deregulated signaling by ErbB1 and ErbB2 occurs in many human malignancies. In contrast, the role ErbB4 plays in tumorigenesis has not been clearly defined. Multiple studies have suggested that increased ErbB4 signaling couples to uncontrolled cell proliferation and metastatic disease. Others have suggested that ErbB4 signaling regulates cell differentiation, growth suppression or that the loss of ErbB4 signaling couples to tumorigenesis. To study biological responses coupled to ErbB4, we have constructed three constitutively-active ErbB4 mutants that signal in the absence of ligand. Using a fibroblast cell line, we have assayed these constitutively-active receptors for coupling to a loss of contact inhibition and for coupling to increased proliferation, saturation density, and anchorage independence. We have determined that the proliferation of cells expressing these gain-of-function mutants is still inhibited by contact and is not anchorage independent. Moreover, the ErbB4 mutants do not alter cell growth rates or saturation density. Our data appears to reinforce studies of the mammary gland that suggests ErbB4 couples to terminal differentiation. Indeed, our results suggest that ErbB4 may act as a tumor suppressor or limit coupling to metastatic disease.

CHAPTER I
INTRODUCTION

Cellular environments are continuously changing to influence the activity of cells. Characterizing how cells interpret and respond to these signals is essential for understanding biological systems. Cell surface receptors mediate the physiological responses from the surrounding microenvironment by transducing signals that govern growth, migration, differentiation, and death (Schlessinger *et al.,* 2000). Hormones, neurotransmitters, and growth factors act through these cell surface receptors to stimulate protein kinase cascades that dictate physiological responses. Often, deregulated signaling by these receptors leads to tumor pathogenesis. Undoubtedly, understanding how cell surface receptors function will contribute to better therapeutic strategies to limit human disease.

ErbB4 (HER4/p180^{erbB4}) is a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinases (RTK), which includes EGFR/ErbB1/HER1, ErbB2/HER2/Neu, and ErbB3/HER3 (see Figure 1.1). A number of different tissues of epithelial, mesenchymal, and neuronal origin express these receptors (ErbB receptors). Numerous studies have demonstrated that ErbB receptors play important roles in regulating and specifying fundamental cellular processes including differentiation, proliferation, and development (Jones *et al.,* 1996; Jones *et al.,* 1999; Schroeder and Lee, 1998). The functional roles of ErbB receptors, and ErbB4 in particular, have been well documented in genetically modified mice lacking individual receptors. Mice null for EGFR/ErbB1 have impaired epithelial development in several organs, including skin, lung, and the gastrointestinal tract (Miettinen *et al.,* 1995; Threadgill *et al.,* 1995; Sibilia and Wagner, 1995). ErbB2 null mice die at midgestation (E10.5) due to malformation of myocardial trabeculae (Lee *et al.,* 1995). ErbB3 deficient mice lack Schwann-cell

development in the peripheral nervous system, affecting axons of sensory and motor neurons (Riethmacher *et al.,* 1997). Embryos homozygous for the disruption of the *neuregulin 1* gene (NRG1, an ErbB ligand) or the *erbb4* gene display similar developmental defects as ErbB2 null mice (Meyer and Birchmeier, 1995; Gassmann *et al.,* 1995). Indeed, the absence of cardiac ventricular trabeculation is a prominent feature of ErbB4 null mice. Normal formation of ventricular trabeculae is essential for the homeostasis of blood flow during early stages of heart development. Embryonic lethality results from trabecular malformation, which causes irregular blood flow and defective cardiac function. Overall, homozygous mice defective in NRG1, ErbB2, ErbB3, or ErbB4 die *in utero* between 10 and 14 days following fertilization. Clearly, ErbB receptors play essential roles in modulating the proper development of cardiac muscle and the nervous system.

The physiological roles of ErbB receptors in adult tissues are much less defined. Direct analysis of receptor function has been limited by the embryonic lethality of animals lacking these receptors (Gassmann *et al.,* 1995; Lee *et al.,* 1995; Meyer and Birchmeier, 1995; Riethmacher *et al.,* 1997). In spite of the limitations, ErbB receptor expression patterns in adult tissues reveal that all four ErbB receptors are coordinately expressed during early, mid, and late pregnancy in the mammary glands of mice (Schroeder and Lee, 1998). Interestingly, ErbB1 and ErbB2 are expressed in glands of virgin mice, whereas ErbB4 is not. ErbB1 and ErbB2 display similar levels of expression in puberty and involution (Darcy *et al.,* 2000). Higher expression levels of ErbB4 are seen during late pregnancy and early lactation where epithelial differentiation predominates over proliferation (Jones *et al.,* 1999). ErbB4 is predominantly expressed in mature females, suggesting functional roles in differentiation (Darcy *et al.,* 2000). The coordinate expression of ErbB receptors is not unique to the mammary gland. Pancreatic islet development and ductal growth also appears to depend on multiple ErbB family members (Kritzik *et al.,* 2000).

Ligands for the ErbB family receptors are also coordinately expressed. Epidermal growth factor (EGF), transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF) and neuregulin (NRG) are the predominate ligands associated

with mammary morphogenesis (Yang *et al.*, 1995; Jones *et al.*, 1996). In the mammary gland, the expression of NRG correlates with ErbB3 and ErbB4 levels, whereas expression of TGF-α and HB-EGF parallel the expression of ErbB1 and ErbB2. Functionally, all EGF ligands are capable of activating more than one ErbB receptor. For example, neuregulin binds either ErbB3 or ErbB4 to induce receptor heterodimerization with ErbB2 (Riese *et al.*, 1995). Whether the biological activity associated with neuregulin expression occurs through ErbB2:ErbB3 or ErbB2:ErbB4 heterodimers is unclear. Indeed, the complexity of receptor heterodimerization and the ability of ErbB family members to bind multiple ligands have limited the discovery of functional roles played by each receptor in adult tissues.

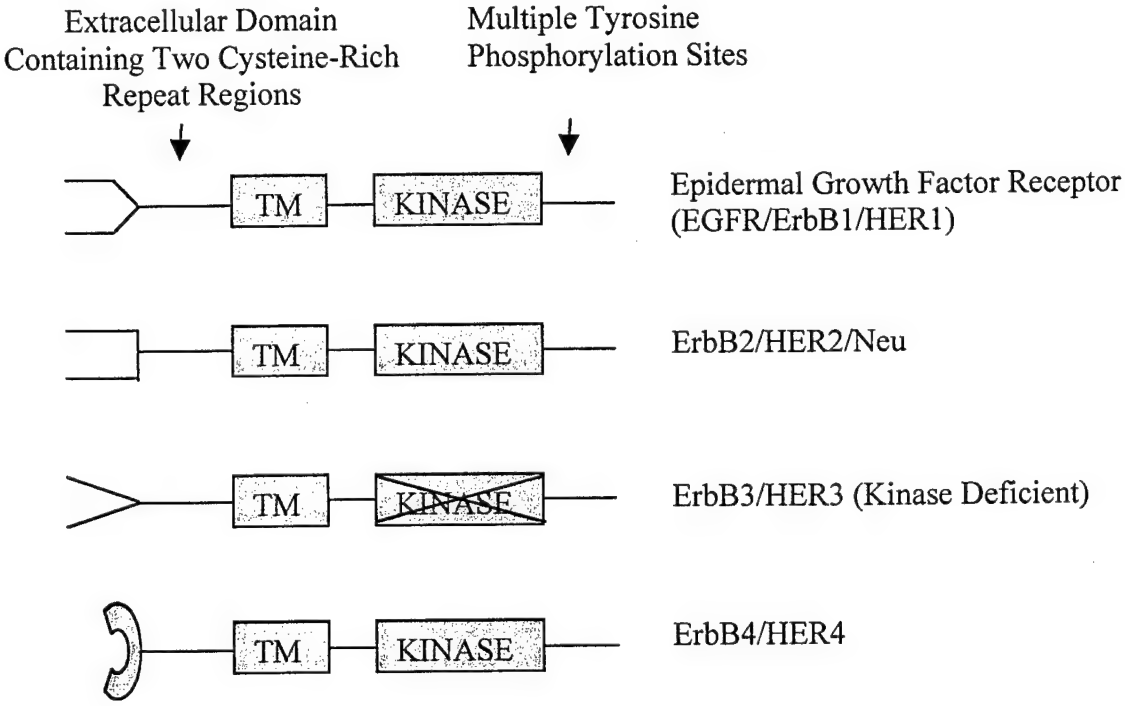

Figure 1.1 **The ErbB family of growth factor receptors.** ErbB1, ErbB2, and ErbB4 all contain a highly conserved intrinsic tyrosine kinase domain (KINASE) and hydrophobic transmembrane (TM) regions. ErbB3 has impaired kinase activity due to substitutions in critical residues in its kinase domain (indicated by the X). ErbB2 is an orphan receptor having no known ligand.

Deregulated expression and/or signaling of ErbB family members occurs in many human malignancies. In particular, ErbB1 or ErbB2 are frequently overexpressed in breast, lung, prostate, ovarian, colon, and pancreatic cancers (Tang and Lippman, 1998; Hynes and Stern, 1994). ErbB1 and ErbB2 overexpression correlates with tumor aggressiveness and poor prognosis for survival (Slamon *et al.,* 1987). There is, however, no detailed analysis of the biological roles played by ErbB4 in metastatic disease.

Although the functional roles ErbB4 plays are unknown, its overexpression has been associated with favorable prognosis in breast cancer (Bacus *et al.,* 1994; Knowlden *et al.,* 1998). A strong positive correlation exists between ErbB4 expression and two favorable prognostic markers for breast cancer, estrogen (ER) and progesterone (PR) receptor expression (Bacus *et al.,* 1996). In contrast, ErbB2 overexpression varies inversely with both PR and ER levels and indicates tumors that are more likely to be metastatic and fatal. The prognostic significance of ErbB4 expression in tumors may also depend on which ErbB family members are co-expressed with ErbB4. In the case of childhood meduloblastoma (one of the most common solid tumors of childhood), patients with tumors overexpressing both ErbB2 and ErbB4 have a significantly worse prognosis for survival than patients with tumors that overexpress either receptor alone (Gilbertson *et al.,* 1997). Finally, loss of ErbB4 expression has been seen in 40-80% in one study of nine solid tumor types of various malignancies, including breast, prostate, and up to 100% in squamous cell carcinomas of the head and neck (Srinivasan *et al.,* 1998). These results raise the intriguing possibility that ErbB4 is unique to this family of receptors. The loss of ErbB4 expression in metastatic disease and the correlation of ErbB4 expression to favorable outcomes in cancer suggest that ErbB4 signaling may limit tumorigenesis. Nonetheless, whether ErbB4 plays roles in differentiation, tumor suppression, or proliferation is still unclear.

Initial efforts to elucidate ErbB4 function have been hampered by many factors. First, there are no known agonists or antagonists specific to the ErbB4 receptor. All of the peptide hormones of the Epidermal Growth Factor (EGF) family that are capable of binding ErbB4 also bind at least one other ErbB family member. For example, epiregulin (EPR) and betacellulin (BTC) activate both ErbB1 and ErbB4 (see Figure 1.2; Riese *et*

al., 1996a; Riese *et al.,* 1998). Receptor signaling by ErbB proteins is triggered by at least 10 different members of the EGF family of polypeptide hormones, including epidermal growth factor (EGF), amphiregulin (AR), transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), and several differentially-spliced variants of neuregulin (NRG). These ligands are capable of activating all ErbB receptors, *in trans* (transmodulation), through ligand-induced receptor heterodimerization (see Figure 1.3; Alroy and Yarden, 1997). For example, EGF stimulates ErbB2 signaling through heterodimerization of ErbB2 with EGFR/ErbB1 (Stern and Kamps, 1988). Yet, EGF will not activate ErbB2 in the absence of EGFR/ErbB1. Consequently, ligands that bind ErbB4 (NRG, BTC, EPR) also stimulate signaling by ErbB1, ErbB2, and ErbB3 (Riese *et al.,* 1996a; Riese *et al.,* 1996b). As a result, a single ErbB ligand stimulates signaling by multiple ErbB receptors. Finally, examination of each ErbB receptor reveals a distinct pattern of carboxyl-terminal phosphorylation sites responsible for coupling to downstream adapter proteins (see Appendix A). As a result, signaling elicited by a receptor heterodimer is not simply the activation of an individual dimerization partner, but rather a unique property acquired by the heterodimer. Thus, the abundance of signaling diversification by ErbB receptors has hampered identifying the biological roles played by each receptor.

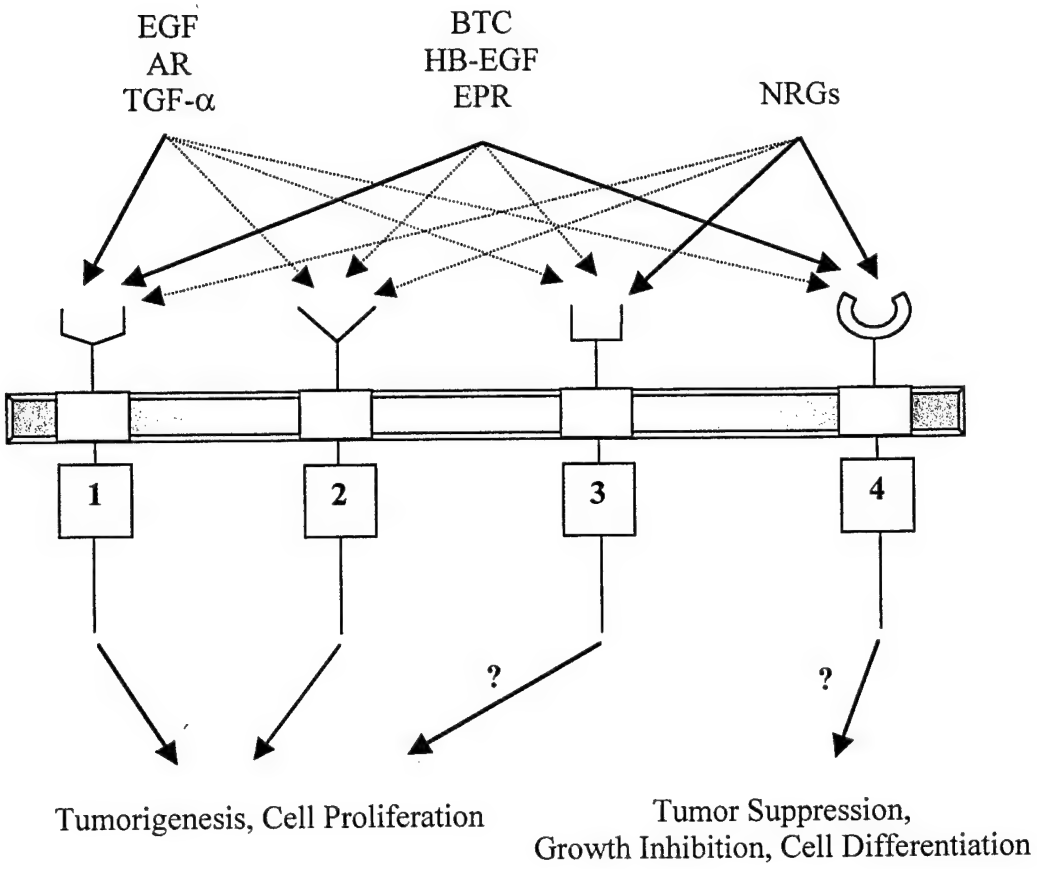

Figure 1.2 **Schematic diagram of ErbB ligands and their associated receptors.** Solid lines represent direct ligand binding, dash lines represent indirect activation of receptors through heterodimerization. EGF, AR, TGF-α bind ErbB1; BTC, HB-EGF, and EPR bind ErbB1 and ErbB4; NRGs bind ErbB3 and ErbB4. ErbB1 and ErbB2 have been demonstrated to couple to tumorigenesis and cell proliferation. The roles for ErbB3 and ErbB4 are less defined and only suggestive.

Structure and Activation of ErbB Receptors

ErbB receptors belong to the type I superfamily of receptor tyrosine kinases on the basis of sequence similarities and structural organization (Ullrich and Schlessinger, 1990). These receptors all possess glycosylated extracellular domains containing two cysteine-rich repeat sequences, a single hydrophobic transmembrane region, a cytoplasmic region containing the catalytic kinase domain, and a carboxyl-terminal cytoplasmic tail with tyrosine phosphorylation sites.

Activation of growth factor receptor signaling is typified by ligand-induced receptor dimerization and subsequent autophosphorylation across receptor dimers on specific carboxyl-terminal tyrosine residues (Schlessinger, 2000). Consequently, phosphorylated tyrosine residues provide docking sites for downstream signaling molecules possessing Src homology (SH2) domains or phosphotyrosine binding (PTB) domains (Cohen *et al.,* 1996a; Sepp-Lorenzino *et al.,* 1996; Crovello *et al.,* 1998). These include adapter proteins like Shc, Gab1, Grb2, Grb7, Crk, and kinases, such as Src and phosphatidylinositol 3'-kinase (PI3K; Prenzel *et al.,* 2001). In addition, ErbB signaling is mediate by protein tyrosine phosphatase SHP1 (Keilhack *et al.,* 1998). The molecules bound to the receptor activate signaling cascades, such as the mitogen-activated protein kinase (MAPK) pathway, protein kinase C (PKC), and the Akt pathway, resulting in coupling to transcription factors that include fos, jun, myc, Sp1, and Egr1 (Yarden and Sliwkowski, 2001). These transcription factors bind specific DNA sequences to affect the expression of multiple target genes (see Appendix B).

Activated receptor complexes have largely been studied in cells engineered to express ErbB receptor family members singly or in pairwise combinations (Riese *et al.,* 1995; Riese *et al.,* 1996a; Riese *et al.,* 1996b; Riese *et al.,* 1998). In cells expressing multiple ErbB family members, extensive receptor-receptor interactions constitute a signaling network whose potential for specification of diverse biological responses is enormous. To limit the complications associated with heterodimeric complexes, we have chosen to study ErbB4 function by expressing receptor mutants that are predicted to form only homodimers and not heterodimers.

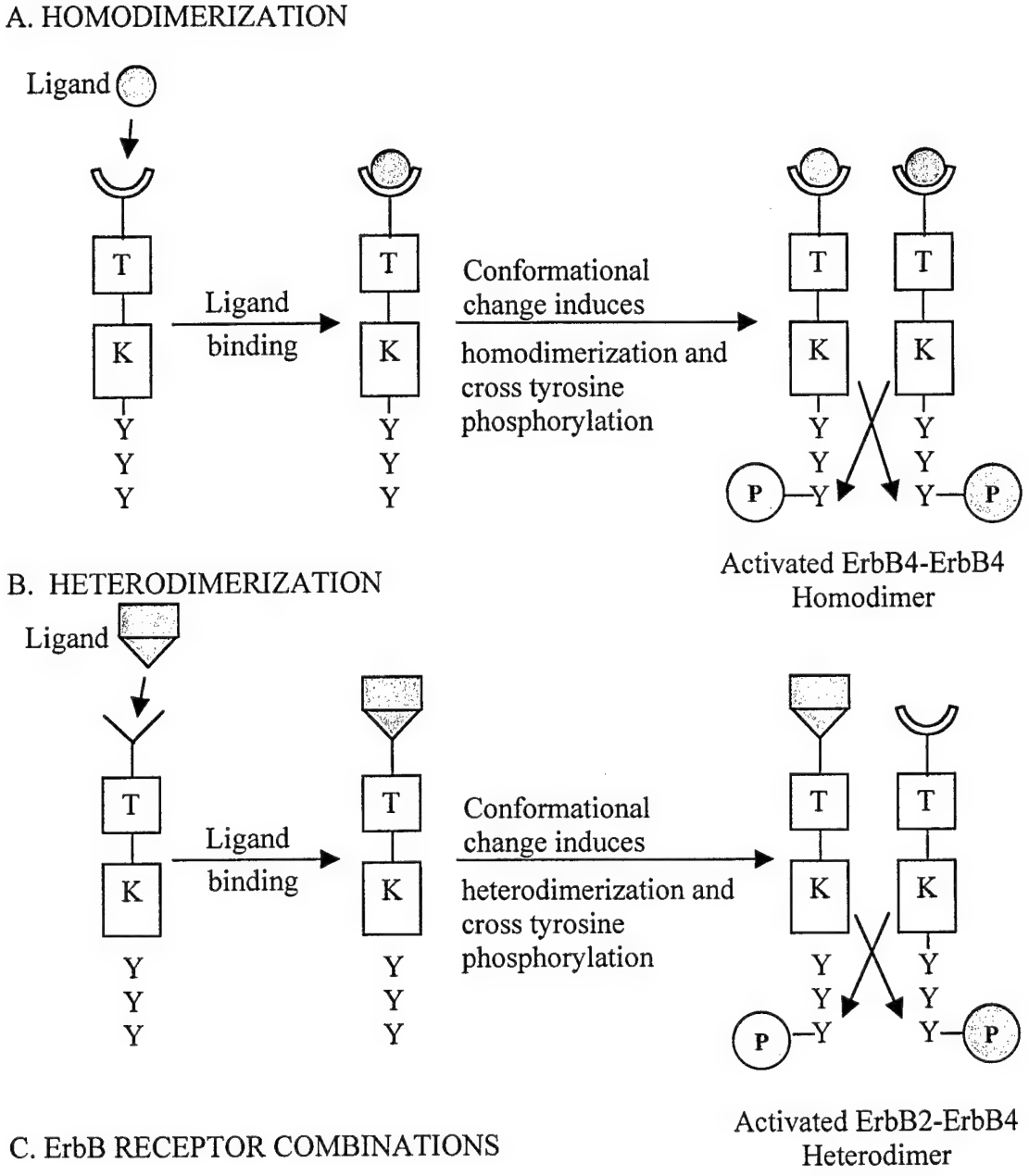

Figure 1.3 **Model for ligand-induced activation of ErbB receptors.** Ligand binding causes a conformational change to allow receptor dimerization and tyrosine Phosphorylation across the dimer. A. Homodimerization of two identical ErbB receptors (ErbB4 shown). B. Heterodimerization of two different ErbB receptors (ErbB2-ErbB4 shown). C. ErbB receptor combinations.

ErbB4

ErbB4 encodes a protein of 1,308 amino acids containing the characteristic organization of a receptor tyrosine kinase ((Plowman *et al.,* 1993; see Figure 1.4). ErbB4 shares a high degree of homology with the other members of the ErbB family. The ErbB4 gene is localized on the distal region of the long arm of chromosome 2 (Zimonjic *et al.,* 1995). Recently, multiple ErbB4 isoforms that vary in the extracellular juxtamembrane or cytoplasmic domain regions have been shown to be differentially expressed *in vivo* (Sawyer *et al.,* 1998; Elenius *et al.,* 1997). This may contribute to the conflicting results concerning ErbB4 function.

The presence of multiple ErbB4 isoforms is novel for this family of proteins. The cytoplasmic variants (CYT-1 and CYT-2) have been found in cell lines of normal and malignant breast tissues (Sawyer *et al.,* 1998). Juxtamembrane variants (JM-a and JM-b) are differentially expressed in neuronal, kidney, and heart tissues (Elenius *et al.,* 1997; see Appendix C for ErbB4 isoforms).

The various ErbB4 isoforms differentially couple to cellular responses. For example, the ErbB4 CYT-2 isoform is unable to couple to PI3-K activation, whereas the ErbB4 CYT-1 isoform is capable of binding PI3-K. (Elenius *et al.,* 1999; Kainulainen *et al.,* 2000). Futhermore, the juxtamembrane isoforms differ in their susceptibility to proteolytic cleavage in response to phorbol 12-myristate 13-acetate (PMA). Treatment with PMA prevented the binding of ^{125}I-NRG-β1 to ErbB4 JM-a, but not ErbB4 JM-b (Elenius *et al.,* 1997). Whether these differences in signaling by ErbB4 isoforms are biologically relevant is still unclear and warrants further investigation.

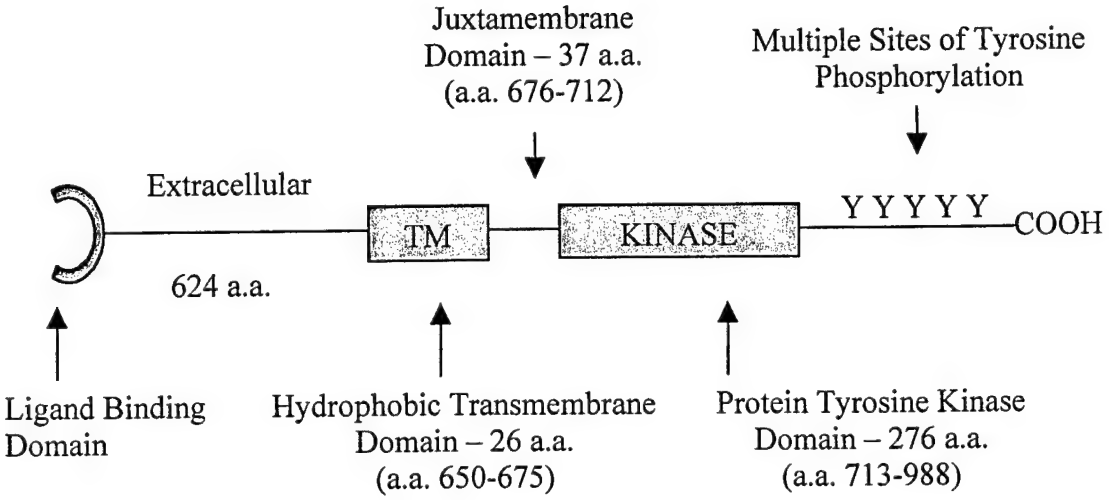

Figure 1.4 **The ErbB4 receptor tyrosine kinase.** The ErbB4 open reading frame encodes 1,308 amino acids with a predicted M_r of 180,000. The ErbB4 receptor contains 18 carboxyl-terminal tyrosine residues with four sites homologous to EGFR autophosphorylation sites (Y1162, Y1188, Y1258, Y1284). ErbB4 contains all 50 cysteine residues conserved in the extracellular domain of ErbB family receptors and contains 11 potential sites for N-linked glycosylation.

Strategy For Studying ErbB4 Receptor Function

As mentioned earlier, it is difficult to use ErbB4 ligands to study ErbB4 function. Consequently, we have opted to generate constitutively-active ErbB4 alleles through the introduction or elimination of extracellular cysteine residues. This is predicted to result in constitutively-dimerized and -active ErbB4 mutants. Introducing cysteine residues to form covalent dimeric receptors is not novel. This technique has been utilized in studying ErbB1 activation. Introducing cysteine residues in the ErbB1 extracellular juxtamembrane region results in the cross-linking of two ErbB1 receptors via disulfide bond formation. These disulfide mediated dimers, in the case of ErbB1, were formed only when EGF was present (Sorkin *et al.,* 1994). Once formed, these constitutive dimers were stable after EGF removal and represented the activated state of the receptor.

Much of our approach to elucidating the biological activities of ErbB4 has come from previous studies on the ErbB2/Neu proto-oncogene. A constitutively-active ErbB2* allele differs from wild-type ErbB2 by only a single substitution in the transmembrane domain (Glu for Val; Bargmann and Weinberg, 1988). The introduction of the valine residue within the transmembrane domain of ErbB2 results in constitutive dimerization, increased kinase activity, and increased oncogenic potential. Although this single mutation is not predicted to form covalent bonds as in the case with our mutants, it is believed that the biological activity of ErbB2* is enhanced by a specific intermolecular interaction that is important in receptor activation (Bargmann and Weinberg, 1988). Furthermore, mutations that eliminate cysteine residues in the extracellular region of the wild-type ErbB2 receptor results in constitutive ErbB2 dimerization and signaling (Siegel and Muller, 1996).

Mutations that either destroy or create cysteine residues in the extracellular region of Fibroblast Growth Factor Receptor 1 (FGFR1), FGFR2, and FGFR3 arise in many craniosynostosis and skeletal syndromes (Webster and Donoghue, 1997). Thanatophoric dysplasia type I (TDI) is a neonatal lethal dwarfism resulting from covalent FGFR homodimers that exhibit increased tyrosine phosphorylation, *in vitro* kinase activity, and biological signaling (d'Avis *et al.,* 1998). Crouzon syndrome is characterized by novel cysteine substitutions within the immunoglobulin-like domain 3 (Ig3), resulting in

constitutive FGFR signaling leading to craniosynostosis, shallow eye orbits, maxillary hypoplasia, and ocular proptosis (Galvin *et al.,* 1996). The constitutive activity of these receptors is a direct result of cysteine residue substitutions in their extracellular domains.

Normally, cysteine residues are predicted to form intramolecular disulfide bonds that are critical for folding, stability, and oligomerization of many glycoproteins (Braakman *et al.*, 1992). The introduction of a cysteine residue is predicted to result in disulfide bonds that align receptor monomers in a dimerized configuration that is productive for signaling (Burke and Stern, 1998). Once aligned, these constitutive complexes mimic the ligand-activated dimeric state of the receptor and provide a powerful functional tool for studying receptor signaling. Furthermore, the elimination of a cysteine residue that might normally participate in intrareceptor disulfide bonds would result in unpaired cysteines that would bond with cysteines on neighboring receptors (interreceptor bonds) to form dimeric complexes.

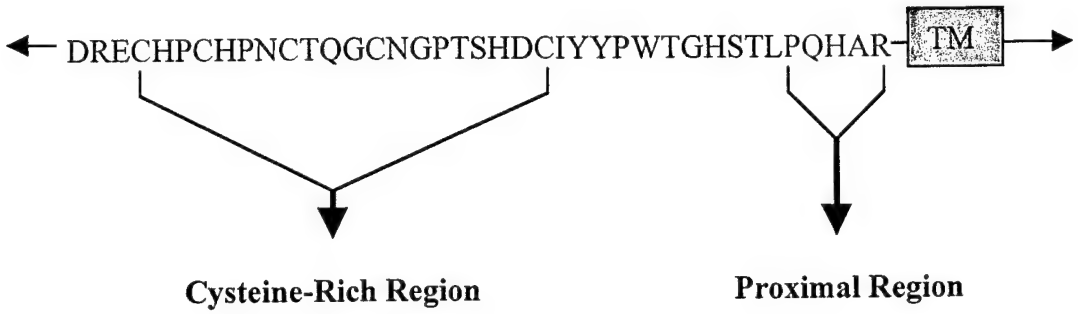

Figure 1.5 **Extracellular region of the ErbB4 receptor.** The cysteine-rich region is comprised of five cysteine residues in the wild-type receptor that were individually mutated to serine. The proximal region contains five wild-type residues that were individually mutated to cysteine.

In this work, we have generated both of these types of mutations in an attempt to generate constitutively-active ErbB4 mutants. We have produced ten distinct ErbB4 receptor mutants that create or destroy cysteine residues. Our focus is on the extracellular region comprising the cysteine-rich and the proximal regions near the transmembrane (see Figure 1.5 and Figure 1.6).

We predict that mutations in the extracellular domain of the ErbB4 receptor that either create or eliminate cysteine residues will generate constitutively-active ErbB4 homodimers. This will enable us to determine biological responses associated with ErbB4 signaling.

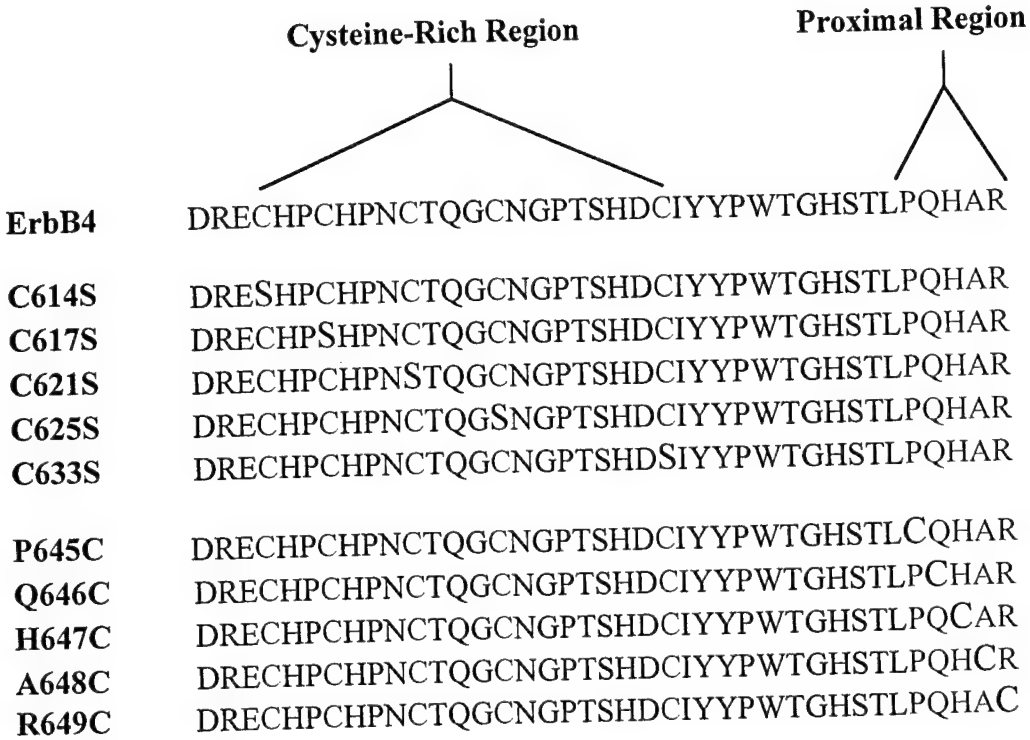

Figure 1.6 **ErbB4 site-directed mutants.** The portion of the ErbB4 extracellular domain that was mutated is shown. Blue residues indicate the residues that were mutated. Red residues indicate the substitutions that were made. The serine substitutions were made in a cysteine-rich region that encompasses amino acid 614 through 633. Cysteine substitutions were made in the proximal region that encompasses amino acids 645 through 649.

CHAPTER II
MATERIALS AND METHODS

Antibodies and Reagents

Anti-ErbB4 mouse monoclonal (SC-8050), anti-ErbB4 rabbit polyclonal (SC-283), and anti-ErbB2 rabbit polyclonal (C-18) antibodies were purchased from Santa Cruz Biotechnology. Goat anti-mouse and goat anti-rabbit horseradish peroxidase conjugated enzymes were purchased from Pierce. Enhanced chemiluminescence (ECL) Western blotting reagents, Redivue™ adenosine 5'-[γ-^{32}P] –triphosphate, and Protein-A Sepharose (CL-4B) were purchased from Amersham Pharmacia Biotech. Anti-phosphotyrosine antibodies were purchased from Upstate Biotechnology. Unless otherwise stated, all other reagents were purchased from Sigma Chemical Company.

ErbB4 Mutagenesis

The recombinant retroviral vector pLXSN-ErbB4 (Riese *et al.,* 1995) was used as the template for site-directed mutagenesis (QuikChange™ Site Directed Mutagenesis Kit, Stratagene) to construct putative gain-of-function, constitutively-active ErbB4 mutants. The mutants were constructed by substituting a novel cysteine in the region of the extracellular domain proximal to the transmembrane domain (amino acids 645, 646, 647, 648, 649). Proximal region mutants are as follows: P645C, Q646C, H647C, A648C, R649C. A second approach involved mutating individual wild-type cysteine residues located in the cysteine-rich region of ErbB4 (amino acids 614, 617, 621, 625, 633) to serine. Cysteine-rich region mutants are as follows: C614S, C617S, C621S, C625S, C633S. Ten total pLXSN-ErbB4-Neo^{R} mutants were constructed: C614S, C617S, C621S, C625S, C633S, P645C, Q646C, H647C, A648C, and R649C. A new restriction

enzyme site was also engineered in each mutant for identification purposes. The new restriction enzyme sites are listed on the Top (T) primer strands with the following sets of primers (T and B) used in site directed mutagenesis:

C614ST Pvu I @ 3812

5' – TATGCTGATCCCGATCGGGAGAGCCACCCATGC – 3'

C614SB

5' – GCATGGGTGGCTCTCCCGATCGGGATCAGCATA – 3'

C617ST Mun I @ 3836

5' – GAGTGCCACCCAAGCCATCCCAATTGCACCCAAGGG – 3'

C617SB

5' – CCCTTGGGTGCAATTGGGATGGCTTGGGTGGCACTC – 3'

C621ST XcmI @ 3852

5' – TGCCATCCAAACAGCACCCAAGGGTGTAATGGTCCCACTAGT – 3'

C621SB

5' – ACTAGTGGGACCATTACACCCTTGGGTGCTGTTTGGATGGCA – 3'

C625ST XcmI @ 3852

5' – TGCACCCAAGGGAGTAATGGTCCCACTAGT – 3'

C625SB

5' – ACTAGTGGGACCATTACTCCCTTGGGTGCA – 3'

C633ST Age I @ 3894

5' – CCCACTAGTCATGACTCCATTTACTACCCATGGACCGGTCATTCCACTT

TACCA – 3'

C633SB

5' – TGGTAAAGTGGAATGACCGGTCCATGGGTAGTAAATGGAGTCATGACTA

GTGGG – 3'

P645CT Age I @3894

5' – ATTTACTACCCATGGACCGGTCATTCCACTTTATGCCAACATGCTAGA

ACTCCC – 3'

P645CB

5' – GGGAGTTCTAGCATGTTGGCATAAAGTGGAATGACCGGTCCATGGGTA

GTAAAT – 3'

Q646CT Age I @ 3894

5' – TACTACCCATGGACCGGTCATTCCACTTTACCATGCCATGCTAGAACTC

CCCTG – 3'

Q646CB

5' – CAGGGGAGTTCTAGCATGGCATGGTAAAGTGGAATGACCGGTCCATGGG

TAGTA – 3'

H647CT Age I @ 3894

5' – CATTTACTACCCATGGACCGGTCATTCCACTTTACCACAATGTGCTAGAC

TCCCCT – 3'

H647CB

5' – AGGGGAGTTCTAGCACATTGTGGTAAAGTGGAATGACCGGTCCATGGGTAG

TAAATG – 3'

A648CT BseRI @ 3919

5' – TCCACTTTACCACAACATTGTAGAACTCCTCTGATTGCAGCTGGA – 3'

A648CB

5' – TCCAGCTGCAATCAGAGGAGTTCTACAATGTTGTGGTAAAGTGGA – 3'

Each site directed mutagenesis reaction contained the following: 5 µl of 10X reaction buffer (supplied with kit), 50 ng of pLXSN-ErbB4, 125 ng of the appropriate "T"-type primer, 125 ng of the appropriate "B"-type primer, 1 µl of dNTP mix (supplied with kit), 1 µl Pfu Turbo DNA polymerase (2.5 U/µl), and dIH_2O to a final volume of 50 µl. Reactions were overlaid with 30 µl of mineral oil and the reaction parameters were as follows: Step #1: 95°C for 30 seconds. Step #2: 95°C for 30 seconds, 55°C for 1 minute, and 68°C for 20 minutes. Step #2 was repeated 18 times. The reactions were then incubated on ice for 10 minutes. After chilling, 1 µl of Dpn I (NewEngland Biolabs) was added to the aqueous layer and the reactions were incubated at 37°C for 1 hour. Standard techniques (Sambrook and Russell, 2001) were utilized for *E. coli* transformation by electroporation. Small scale plasmid DNA preparations were generated using the Wizard® Plus Miniprep DNA purification system (Promega). Minipreps were screened for the new restriction enzyme site and *E. coli* harboring the plasmid with the appropriate mutations were expanded and large-scale plasmid isolation and purification were performed using standard techniques (Sambrook and Russell, 2001). The presence of the appropriate mutations and the absence of any additional mutations were confirmed by DNA sequencing.

Cell Lines

The Ψ2, PA317, and FR3T3 cell lines were gifts from Daniel DiMaio, Yale University. All cell lines were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 IU/ml penicillin, 50 μg/ml streptomycin (Mediatech), and 0.25 μg/ml fungizone (Amphotericin B; Gibco/BRL). Recombinant cell lines generated in the course of the following experiments were propagated in the media described above supplemented with 200 μg/ml G418 (Mediatech).

Retroviral Infections

The retroviral vector pLXSN (RV-Neo^R) is derived from the Moloney Murine Sarcoma Virus, and the Moloney Leukemia Virus, and was obtained from Dan DiMaio, Yale University. pLXSN contains an SV40 early promoter that controls the transcription of the aminoglycoside 3'-phosphotransferase gene (Neo^R gene) and the Ψ packaging signal (Miller and Rosman, 1989). The Neo^R gene confers resistance to the aminoglycoside antibiotic G418 (geneticin, Gibco/BRL). In eukaryotic cells G418 interferes with the function of the 80S ribosome and kills cells by blocking protein synthesis (Southern and Berg, 1982).

The ψ2 ecotropic retrovirus packaging cell line (Mann *et al.,* 1983) was transfected for stable expression of the pLXSN-ErbB4 mutants using standard techniques (Sambrook and Russell, 2001). These transfected Ψ2 cells were also used to package recombinant retroviruses expressing the Neo^R gene. Retroviral particles were harvested from the media conditioned by the Ψ2 cells and were used to infect PA317 and FR3T3 cell lines. Ψ2 ecotropic retrovirus packaging cell lines produce replication-defective, helper-free retrovirus stocks with titers ranging from 10^3 to 10^6 colony-forming units/ml (Mann *et al.,* 1983; Miller and Buttimore, 1986).

The PA317 amphotropic packaging cell line (Miller and Buttimore, 1986) was infected with the ecotropic recombinant retroviruses harvested from Ψ2 cells. Cells were seeded in 60 mm dishes and were infected when the cells were at approximately 75% confluence. The medium was aspirated from each plate of cells and approximately 200

µl of harvested retrovirus was added to each plate along with 900 µl DMEM supplemented with 5 µg/ml polybrene. Following incubation for 2 hours at 37°C, 4 ml of DMEM containing 2% FBS and 5 µg/ml polybrene was added to each dish. Approximately 16-24 hours later, the cells were detached from the plates with trypsin and suspended in a total volume of 9 ml of fresh medium. The cells were aliquoted into three 100 mm dishes and medium was added to a final volume of 10 ml per plate. Approximately 24 hours later, the medium on these plates was replaced with medium containing 900 µg/ml G418 to select for infected cells. Colonies of drug resistant cells were isolated and pooled into cell lines that were subsequently screened for ErbB4 receptor expression. Media conditioned by the transfected PA317 cells were harvested. They are high-titer amphotropic retrovirus stocks.

The FR3T3 cell line was infected as described earlier, except 200 Cfu (titered in PA317 cells) of each Ψ2 ecotropic retrovirus stock was used instead of 200 µl.

Immunoprecipitation and Immunoblotting

The analysis of ErbB4 and ErbB2 receptor tyrosine phosphorylation by immunoprecipitation and anti-phosphotyrosine immunoblotting has been described previously (Riese *et al.,* 1995; Riese *et al.,* 1998). Briefly, cell lysates were generated and quantified for protein content by Bradford Assay (Coomassie® Protein Assay Reagent-Pierce; Bradford, 1976) and equal amounts of protein extract were immunoprecipitated with antibodies specific for ErbB2 or ErbB4. The samples were resolved by electrophoresis on a 7.5% acrylamide, 0.17% bisacrylamide, 0.1% SDS gel and electrotransferred onto nitrocellulose. The blots were probed with the anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc.) Antibody binding was detected with a goat anti-mouse horseradish peroxidase coupled antibody (1:3,000 in TBS-T) and enhanced chemiluminescence.

The amounts of receptor tyrosine phosphorylation and expression (in PA317 cells) were quantified by digitizing the chemilumigrams using a Linotype-Hell Jade 2-dimensional scanning densitometer set at 600 dpi resolution. The bands on the images

were quantified using NIH Image for Macintosh v1.6 software. Values are expressed as arbitrary units. Background levels were computed using the vector control lanes and were subtracted from the gross values to produce net receptor expression and tyrosine phosphorylation values. The digitized images were also cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software.

Dimerization Assay

Detection of ErbB4 dimer formation was performed as described previously (Siegel and Muller, 1996) except that EBC buffer (Irusta and DiMaio, 1998) containing 10 mM Iodoacetamide, 1 mM Na_3VO_4, and 1% Aprotinin was used for cell lysis and for washing immunoprecipitates. Briefly, cells were lysed in EBC containing Iodoacetamide, Na_3VO_4, and Aprotinin. The protein content of the lysates was quantified by Bradford Assay and ErbB4 receptors were immunoprecipitated from samples containing equal amounts of protein. The immunoprecipitates were then boiled for 5 minutes in SDS loading buffer in the absence of 2- mercaptoethanol (62.5 mM Tris-HCl, 2% SDS, 5% glycerol, 0.25% bromophenol blue), resolved by electrophoresis on a 4-12 % acrylamide-0.19 to 0.56% bisacrylamide gradient gel (Burke and Stern, 1998), and electrotransferred to nitrocellulose at 200 mA overnight. Membranes were probed with an anti-ErbB4 mouse monoclonal antibody (1:300). Antibody binding was detected with a goat anti-mouse horseradish peroxidase coupled antibody (1:2,500 in TBS-T) and enhanced chemiluminescence.

In Vitro Kinase Assay

ErbB2 and ErbB4 were immunoprecipitated from protein extracts from PA317 cells as described earlier (Riese *et al.,* 1995). Immune complex kinase reactions were performed as previously described (Cohen *et al.,* 1996a). Briefly, ErbB2 or ErbB4 was immunoprecipitated from protein extracts using 35 µl Protein-A Sepharose and 5 µl of anti-ErbB2 or anti-ErbB4 rabbit polyclonal antibodies. Samples were washed five times

in 500 µl kinase buffer (20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 3 mM $MnCl_2$). After the last wash, the samples were resuspended in 100 µl kinase buffer supplemented with 10 µCi of [γ-^{32}P]ATP and were incubated for 10 minutes at room temperature. The beads were then washed two times in NET-N (Irusta and DiMaio, 1998), boiled for 5 minutes in 2X Laemmli sample buffer, and the samples were resolved by electrophoresis on an SDS-7.5% acrylamide-0.17% bisacrylamide gel. The gel was then dried overnight and exposed to X-ray film for approximately 20 hours. The autoradiograms were digitized using a Linotype-Hell Jade 2-dimensional scanning densitometer set at 600 dpi resolution. The bands on the images were quantified using NIH Image for Macintosh v1.6 software. Values are expressed as arbitrary units. Background levels were computed using the vector control lanes and were subtracted from the gross values to produce net kinase activity values. The digitized images were also cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software.

Focus Forming Assay

FR3T3 cell were infected with Ψ2 ecotropic recombinant retroviruses as stated earlier and described previously (Burke and Stern, 1998). Approximately 24 hours after infection, cells were split into three 60 mm dishes. Cells were maintained in DMEM supplemented with 10% FBS until foci appeared. The medium was changed every three days. Cells were fixed in methanol and stained with Giemsa (1:25 in PBS; Fisher) to visualize the foci.

Anchorage Independency Assay

FR3T3 cells were seeded at a density of 2 x 10^4 cells in 60 mm dishes containing 2.5 ml of 0.3% Low Melting Point Agarose (LMP-agarose; Gibco/BRL) as described previously (Hwang *et al.*, 1993). Every three days DMEM supplemented with 10% FBS and 0.3% LMP-agarose was added to each plated. The cells were incubated at 37°C for 10 days and fields were photographed with an Olympus OM-10 camera attached to an

Olympus CK-2 phase-contrast inverted microscope. Photographs represent fields from three independent experiments.

Growth Rate/Saturation Density

Stable FR3T3 cell lines expressing the wild-type ErbB4 receptor, ErbB2*, or the ErbB4 mutants (Q646C, H647C, A648C) were plated in ten 60 mm dishes at a density of 2×10^4 cells per dish. Cells were incubated from one to ten days at 37°C. Cells were counted (Coulter Counter® ZM) each day for a total of ten days. The mean and standard error of the mean (SEM) are representative of three independent experiments.

CHAPTER III
RESULTS

Cysteine mutations initially discovered in the neu (ErbB2*) proto-oncogene and fibroblast growth factor receptors demonstrate that a single point mutation in the extracellular region may result in a constitutively-dimerized and -active receptor. We were interested in assessing whether ErbB4 receptors harboring analogous mutations were also constitutively active. To test our hypothesis, cysteine residues were either introduced or eliminated in the extracellular region of the ErbB4 receptor. We constructed ErbB4 mutants in which individual cysteine residues were converted to serine (C614S, C617S, C621S, C625S, C633S). We also constructed ErbB4 mutants that contained a novel cysteine residue (P645C, Q646C, H647C, A648C, R649C).

ErbB4 mutant receptors have increased signaling

Since a single unpaired cysteine residue might interfere with protein folding and export to the cell surface, we were concerned that our mutants might not be stably expressed. We analyzed the expression and tyrosine phosphorylation of the ErbB4 mutants in the Ψ2 ecotropic retrovirus packaging cell line. Ψ2 cells were transfected as described in Chapter II. These cells efficiently express genes from recombinant retroviral vectors.

As shown in Figure 3.1, Ψ2 cells transfected with the retroviral vector control LXSN, or the retroviral vectors containing wild-type ErbB4 (ErbB4 lane), mutant ErbB2* (ErbB2* lane) or the three mutant receptors (Q646C, H647C, A648C) were analyzed for receptor phosphorylation and expression. Cells transfected with vector alone do not exhibit any receptor expression or phosphorylation (LXSN lane). ErbB2* was used as a positive control throughout our experiments because this mutant is

constitutively-dimerized and -active (Bargmann and Weinberg, 1988). Although it is unclear how ErbB2* maintains an activated dimeric complex in conditions that would normally disrupt non-covalent receptor interactions, ErbB2* is constitutively phosphorylated in Ψ2 cells (Figure 3.1 top panel, ErbB2* lane).

Anti-ErbB4 immunoblotting recognizes 180-kDa proteins in Ψ2 cells transfected with retroviral vectors containing wild-type ErbB4 or the three mutant receptor constructs (bottom panel), but not in cells transfected with the vector alone or with retroviral vectors containing ErbB2*. Moreover, levels of ErbB4 expression are roughly equivalent in cells transfected with wild-type ErbB4 or with the ErbB4 mutants (bottom panel).

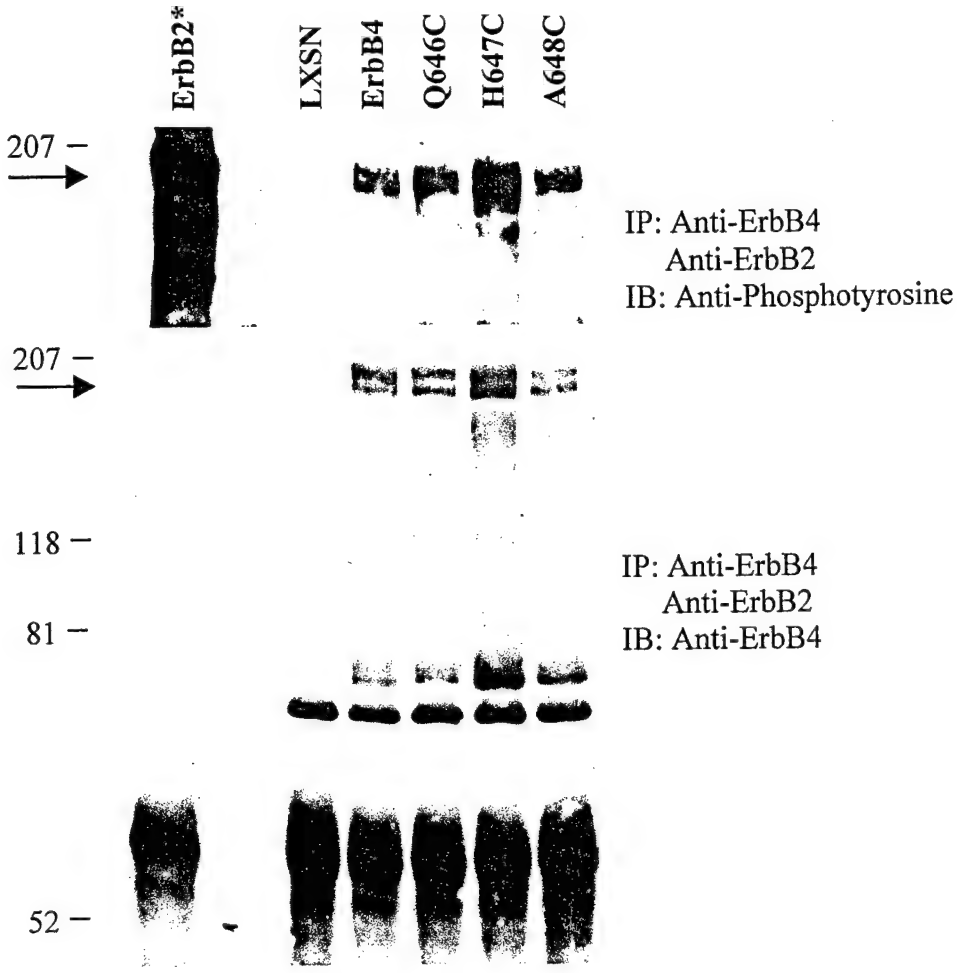

Figure 3.1 **Expression and phosphorylation of ErbB4 mutant receptors in Y2 cells.** Receptors were immunoprecipitated from lysates containing equal amounts of protein (250 μg) prepared from Ψ2 cells transfected with recombinant retroviral vectors containing wild-type ErbB4, ErbB2*, LXSN, or the ErbB4 mutants (Q646C, H647C, A648C). Cells transfected with the LXSN vector serve as a negative control. Immunoprecipitations were resolved by SDS-PAGE, electroblotted to nitrocellulose, and immunoblotted with an anti-phosphotyrosine antibody (top panel). The blot was then stripped and reprobed with an anti-ErbB4 rabbit polyclonal antibody (bottom panel). The arrows indicate bands corresponding to ErbB receptors.

Our initial results demonstrate that the ErbB4 mutants are tyrosine phosphorylated in transfected Ψ2 cells. However, wild-type ErbB4 exhibits a similar level of tyrosine Phosphorylation as the ErbB4 mutant receptors. Previous observations indicate that ErbB4 overexpression results in tyrosine phosphorylation in the absence of ligand (Cohen *et al.*, 1996a). Therefore, the ErbB4 tyrosine phosphorylation seen in Figure 3.1 could be a result of overexpression. To further explore the possibility that the ErbB4 mutants are constitutively active, we harvested ecotropic retroviruses from Ψ2 cell lines for infection of the amphotropic retroviral packaging PA317 cell line.

Retroviral-mediated gene transfer offers a number of advantages over other gene transfer strategies, including high transduction efficiencies and stability of the integrated DNA in the infected cells (Gerstmayer *et al.*, 1999). However, transfection of viral vectors into Ψ2 cells results in ecotropic retroviruses with a limited host range. Moreover, the titers of these retroviral stocks are typically low. Therefore, PA317 cells were infected to produce high-titer amphotropic retroviral stocks and to assay the biochemical properties of the ErbB4 mutants.

PA317 cells infected with retroviruses that direct the expression of wild-type ErbB4 or the ErbB4 mutants were assayed for ErbB4 expression and phosphorylation in Figure 3.2. As expected, cells infected with the LXSN vector control retrovirus do not exhibit ErbB4 expression (right panel) or tyrosine phosphorylation (left panel). Cells infected with wild-type or mutant ErbB4 retroviruses exhibit ErbB4 expression (Figure 3.2, right panel). However, cells infected with the mutant ErbB4 retroviruses exhibit abundant tyrosine phosphorylation, whereas cells infected with the wild-type ErbB4 retrovirus exhibit minimal ErbB4 tyrosine phosphorylation (Figure 3.2, left panel).

Quantification of the chemilumigrams shown in Figure 3.2 suggests that the expression levels of the three ErbB4 mutants is less than three times greater the amount of wild-type ErbB4 expression (Table 3.1). In contrast, the amounts of tyrosine phosphorylation of the three ErbB4 mutants appear to be much greater than the amount of wild-type ErbB4 tyrosine phosphorylation. Moreover, the ratios of ErbB4 tyrosine phosphorylation to ErbB4 expression for the three ErbB4 mutants appear to be at least four times greater than the ratio for wild-type ErbB4. These data suggest that the three

ErbB4 mutants exhibit greater amounts of tyrosine phosphorylation on a per molecule basis than does wild-type ErbB4. This data suggests that the three mutants are constitutively active for signaling. Although, it is unclear if the increased tyrosine phosphorylation seen in the ErbB4 mutants is caused by increased half-life of the protein on the cell surface. Indeed, if the receptors are not down regulated from the cell surface the activated state of the receptor might be maintained in an active configuration for signaling.

Table 3.1 **The Q646C, H647C, and A648C ErbB4 mutants exhibit increased normalized tyrosine phosphorylation.**

Cell Line	**ErbB4 Tyrosine Phosphorylation**	**ErbB4 Expression**	**Ratio**
Wild-type ErbB4	212867	1831340	0.116
ErbB4 Q646C	1860472	3329432	0.558
ErbB4 H647C	2944443	4659376	0.631
ErbB4 A648C	3981658	4509147	0.883

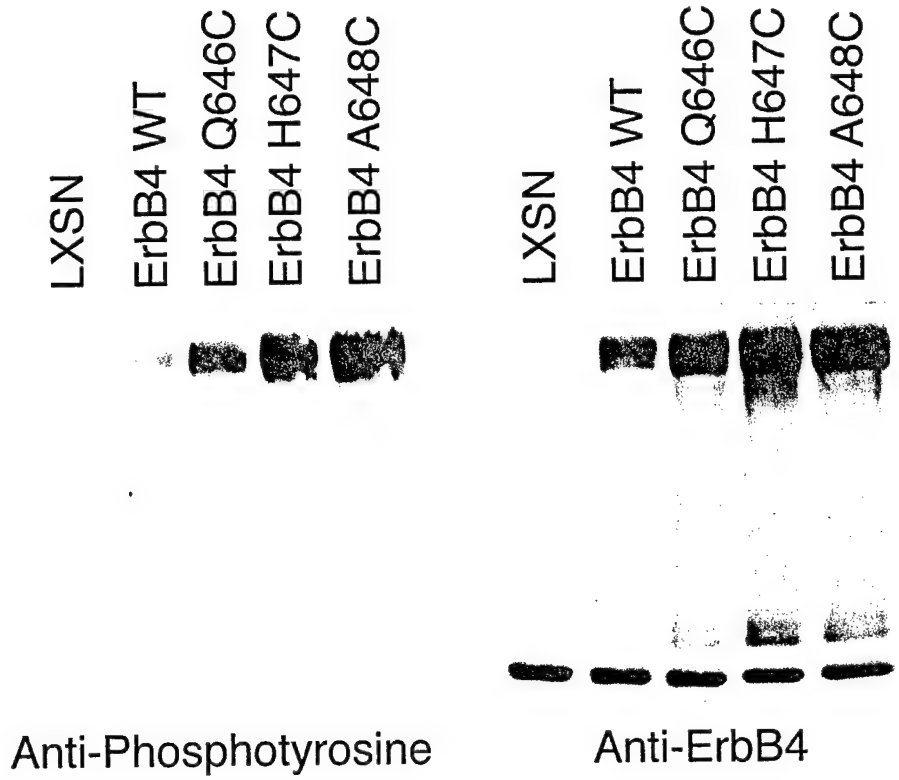

Figure 3.2 **ErbB4 mutants are constitutively tyrosine phosphorylated.** ErbB4 expression and tyrosine phosphorylation was assayed in PA317 cells infected with retroviruses that direct the expression of wild-type ErbB4 or the ErbB4 mutants. Cells infected with the LXSN recombinant retrovirus served as the negative control. Lysates were prepared from each of the cell lines and ErbB4 was immunoprecipitated from 1000 μg of each lysate. Samples were resolved by SDS-PAGE, electroblotted to nitrocellulose, and immunoblotted with an anti-phosphotyrosine antibody (left panel). The blot was then stripped and probed with an anti-ErbB4 rabbit polyclonal antibody (right panel). ErbB4 is represented by the band at the top of the blots.

Corresponding titers were determined for each retroviral stock to analyze the efficiency of infection. Ecotropic retrovirus stocks were titered by infecting FR3T3 cells and counting colonies of cells resistant to G418. Table 3.2 summarizes the titers obtained in colony-forming units per ml of retroviral stock. No significant differences in retroviral titers were observed to account for the difference in wild-type ErbB4 expression as compared to the ErbB4 mutants in the PA317 cell lines. These retroviral titers confirm that similar numbers of infectious particles were used to generate the PA317 cell lines. Therefore, we have concluded that the increased tyrosine phosphorylation of the three ErbB4 mutants reflects the increased signaling activity of these mutants rather than simple gross overexpression of the mutants.

Table 3.2 **Titers of Ψ2 retroviral particles**. Approximately 200 Cfu of each ecotropic retrovirus stock was used to infect 60 mm dishes of subconfluent FR3T3 cells. Approximately 20 hours later, 60 mm dishes were split into three 100mm dishes and allowed to grow for 2 weeks in the presence of DMEM supplemented with 10% FBS and 900 μg/ml G418. The medium was changed every three days and G418 resistant colonies were counted when uninfected cells had died. Ecotropic stocks were titered three times in FR3T3 cells. Standard Error of the Mean (SEM) represents three trials.

Ψ2 Retroviral Stock	Target Cell	Titer (Cfu/ml)	SEM
LXSN	FR3T3	NA	NA
ErbB2*	FR3T3	3.43×10^3	7.3×10^2
ErbB4	FR3T3	4.33×10^3	9.5×10^2
Q646C	FR3T3	1.19×10^3	1.6×10^2
H647C	FR3T3	2.56×10^3	5.6×10^2
A648C	FR3T3	1.79×10^3	4.0×10^2

ErbB4 mutant receptors are constitutively-dimerized

In order to ascertain whether the ErbB4 mutants are constitutively form dimers, we resolved ErbB4 immunoprecipitates by SDS-PAGE in the absence of reducing agents and analyzed the samples by immunoblotting. As shown in Figure 3.3, stable ErbB4 receptor dimers could not be detected in cells infected with the retroviral vector control (LXSN lane) or in cells that were infected with the retroviral vector containing wild-type ErbB4 (ErbB4 lane). ErbB4 is expressed in PA317 cells as shown previously (Figure 3.2; ErbB4 lane), but is not efficiently recognized by the anti-ErbB4 mouse monoclonal antibody used in this experiment (Figure 3.3; ErbB4 lane). It is unclear whether the markedly higher ErbB4 monomeric and dimeric mutant expression levels are due to increased protein stability. Nonetheless, our data suggests that the ErbB4 mutant receptors readily form dimers in the absence of ligand.

To determine whether the receptors active for signaling occur primarily as monomers or as dimers, we prepared samples as described earlier and analyzed them by anti-phosphotyrosine immunoblotting. Figure 3.4 indicates that the tyrosine phosphorylated ErbB4 mutant receptors are both monomeric and dimeric. Moreover, the total amount of tyrosine-phosphorylated ErbB4 was much greater in cells that express the ErbB4 mutants than in cells that express wild-type ErbB4. Furthermore, it is unclear how ErbB2* maintains an activated dimeric complex in conditions that would normally disrupt non-covalent receptor interactions. ErbB2* is not predicted to form disulfide bonds, but is well documented to be constitutively active and dimerized in the absence of ligand (Bargmann and Weinberg, 1988; Stern *et al.*, 1988). Finally, monomeric and dimeric ErbB4 mutants appear to be active for signaling in both the monomeric and dimeric states.

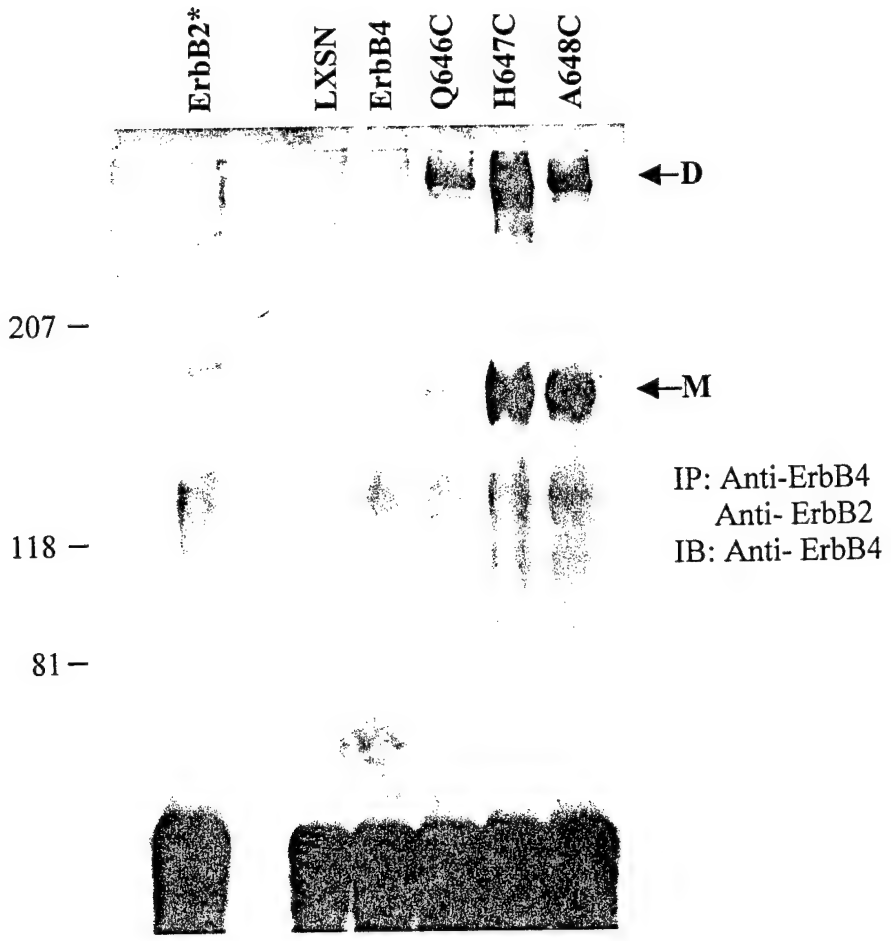

Figure 3.3 **Constitutively-dimerized ErbB4 mutant receptors**. PA317 cells stably expressing wild-type ErbB4, ErbB2*, or the receptor mutants (Q646C, H647C, A648C) were lysed in EBC buffer containing 10 mM Iodoacetamide. ErbB receptors were immunoprecipitated from approximately 2000 μg of cell lysate using anti-ErbB2 (ErbB2* lane only) or anti-ErbB4 rabbit polyclonal antibodies. Immunoprecipitated proteins were resolved under nonreducing conditions by SDS-PAGE on a 4-12% acrylamide gradient gel and electroblotted to nitrocellulose. Immunoblotting was performed with an anti-ErbB4 mouse monoclonal antibody. The arrows indicate the position of monomeric (M) and dimeric (D) ErbB4 receptors.

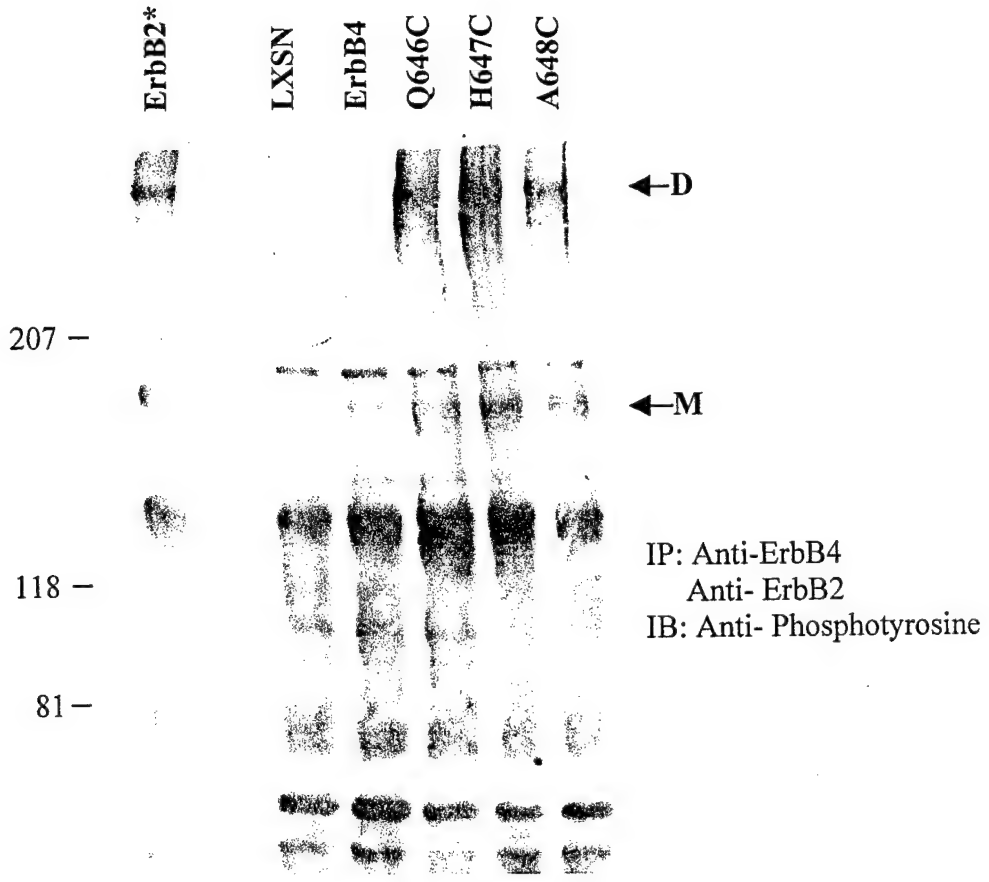

Figure 3.4 **Phosphorylation of constitutively-dimerized ErbB4 mutant receptors.** PA317 cells stably expressing wild-type ErbB4, ErbB2*, or the receptor mutants (Q646C, H647C, A648C) were lysed in EBC buffer containing 10 mM Iodoacetamide. ErbB receptors were immunoprecipitated from approximately 2000 μg of cell lysate using anti-ErbB2 (ErbB2* lane only) or anti-ErbB4 rabbit polyclonal antibodies. Immunoprecipitated proteins were resolved under nonreducing conditions by SDS-PAGE on a 4-12% acrylamide gradient gel and electroblotted to nitrocellulose. Immunoblotting was performed with an anti-phosphotyrosine antibody. The arrows indicate the position of monomeric (M) and dimeric (D) ErbB4 receptors.

ErbB4 mutant receptors have increased *in vitro* kinase activity

We have demonstrated that the Q646C, H647C, and A648C ErbB4 mutants exhibit greater ligand-independent tyrosine phosphorylation than wild-type ErbB4. Next, we assessed whether the increased tyrosine phosphorylation of the mutants correlates with increased kinase activity. To measure the levels of intrinsic kinase activity, equal amounts of protein extracts were immunoprecipitated with an anti-ErbB4 polyclonal antibody. Kinase reactions were then performed in the presence of [γ-^{32}P] ATP. The reaction products were resolved by electrophoresis. The gels were dried and the reaction products were visualized by autoradiography.

In Figure 3.5 we show that PA317 cells infected with the LXSN vector control retrovirus lack detectable ErbB4 kinase activity. Moreover, PA317 cells that express the three constitutively-active ErbB4 mutants exhibit greater ErbB4 tyrosine kinase activity than cells that express wild-type ErbB4. Quantification of the bands on the autoradiogram indicates that the Q646C and H647C ErbB4 mutants exhibit approximately 5-fold more kinase activity than does wild-type ErbB4, whereas the A648C ErbB4 mutant exhibits approximately 9-fold more kinase activity than does wild type ErbB4. Given that the expression of the ErbB4 mutants is somewhat greater than the expression of wild-type ErbB4 (Table 3.1), it appears that the intrinsic kinase activity of the three ErbB4 mutants is approximately 2-fold greater than the intrinsic kinase activity of wild-type ErbB4. This data suggests that the ErbB4 mutants Q646C, H647C, and A648C are constitutively active for signaling.

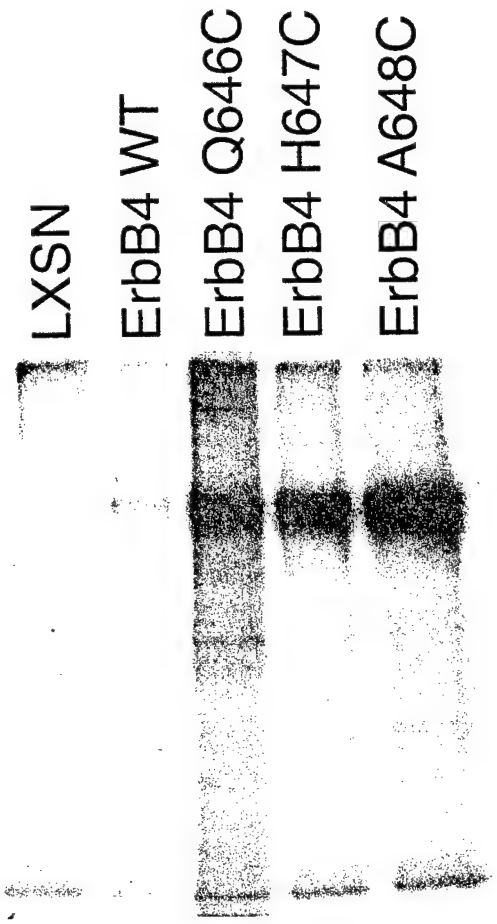

Figure 3.5 **Q646C, H647C, and A648C mutants exhibit increased *in vitro* kinase activity.** Equal amounts of protein lysates from PA317 cells that stably express wild-type ErbB4 or the ErbB4 mutants (Q646C, H647C, A648C) were immunoprecipitated with an anti-ErbB4 rabbit polyclonal antibody. Lysates from PA317 cells that express the LXSN vector served as the negative control. Kinase reactions were performed on the immunoprecipitates in the presence of [γ-^{32}P]ATP. The products were resolved by SDS-PAGE. The gel was dried overnight and exposed to X-ray film for approximately 20 hours to visualize the products of the kinase reactions.

Constitutively-active ErbB4 mutant receptors lack transforming activity

Conflicting results are seen in assays for growth transformation by ErbB4. ErbB4 induces a loss of contact inhibition (foci) in NIH 3T3 clone 7 cells in the absence of ligand (Cohen *et al.*, 1996a). In these cells the loss of contact inhibition was further stimulated with an ErbB4 ligand, neuregulin 2β. In contrast to these results, NIH 3T3 clone 7d cells transfected with wild-type ErbB4 did not form foci in the presence or absence of neuregulin 1β (Cohen *et al.*, 1996b; Zhang *et al.*, 1996).

To try to resolve this dichotomy, we next determined if increased ErbB4 phosphorylation and kinase activity correlates with loss of contact inhibition. FR3T3 rat fibroblasts were infected with 200 Cfu of each ecotropic retrovirus stock (vector, ErbB2*, wild-type ErbB4, Q646C, H647C, A648C) and assayed for focus formation.

FR3T3 cells that express ErbB2* form foci 9 days after infection, whereas cells infected with the vector control did not (Figure 3.6). Moreover, cells that express wild-type ErbB4 or the constitutively-active ErbB4 mutants did not form foci. Eighteen days after infection, cells that express the constitutively-active ErbB4 mutants formed a few foci; however, the number of foci was comparable to those seen when cells express wild-type ErbB4. Thus, we conclude that the constitutively-active ErbB4 mutants do not induce a significant loss of contact inhibition in FR3T3 fibroblasts.

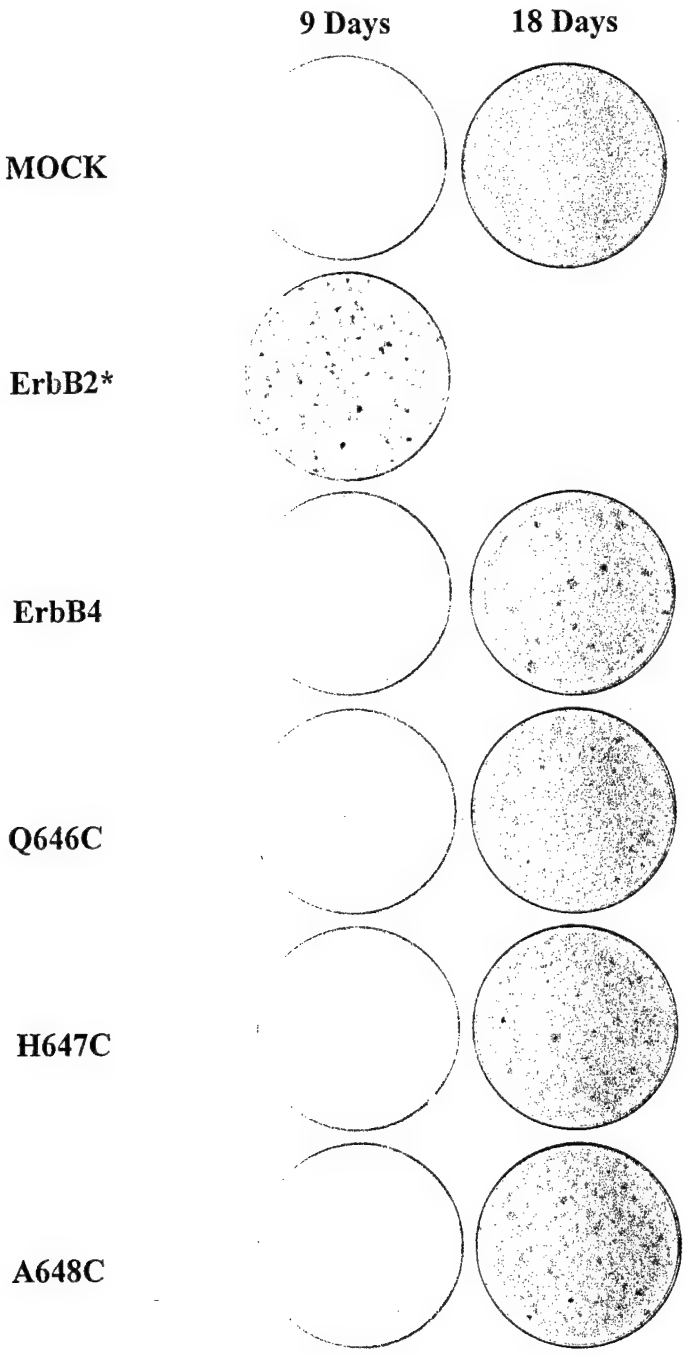

Figure 3.6 **Constitutively-active ErbB4 receptors do not induce a significant loss of contact inhibition**. FR3T3 fibroblasts infected with the vector control retrovirus, the ErbB4 retrovirus, the ErbB2* retrovirus, or the ErbB4 mutant retroviruses were assayed for loss of contact inhibition.

Constitutive ErbB4 signaling does not induce anchorage-independent growth

Next, we investigated whether the increased phophorylation and kinase activity of the constitutively-active ErbB4 mutants induced anchorage-independent growth of FR3T3 fibroblasts. Infected FR3T3 cells were seeded at a density of 2 x 10^4 cell per 60 mm dish in medium containing 0.3% LMP-agarose and cells were incubated for 10 days. Fresh medium (DMEM/10% FBS) containing LMP-agarose was added every three days and photographs were taken of representative fields.

Cells that express ErbB2* exhibit anchorage-independent growth (Figure 3.7). In contrast, cells that were infected with the vector control and cells that express wild-type ErbB4 or the ErbB4 mutants did not exhibit anchorage-independent growth. The results of this assay are consistent with the results of the focus formation assay; both assays indicated that the constitutively-active ErbB4 mutants do not oncogenically transform the growth of FR3T3 fibroblasts.

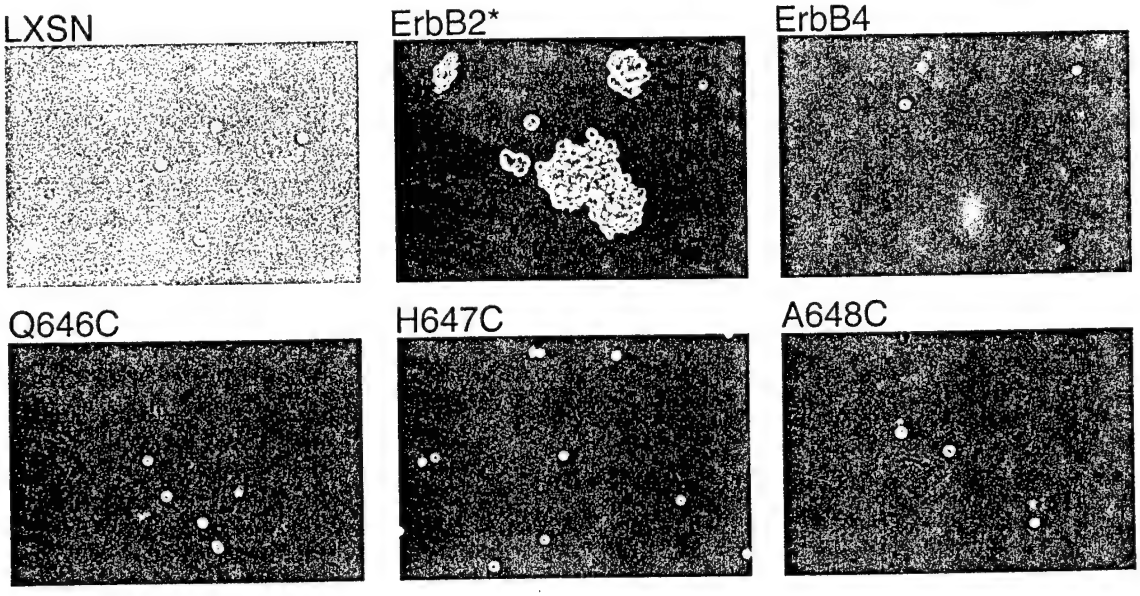

Figure 3.7 **Constitutively-active ErbB4 receptors do not induce growth in soft agar.** Infected FR3T3 cells expressing vector, ErbB2*, or the receptor mutants (Q646C, H647C, A648C) were seeded in semi-solid medium at a density of 2 x 10^4 cell per 60 mm dish. The cells were incubated for 10 days and fields were photographed. Figures shown are representative of three independent experiments.

Constitutive ErbB4 signaling does not increase the growth rate or saturation density of cells

Constitutive ErbB2* signaling is coupled to increased growth rates and saturation densities in fibroblasts (Hynes and Stern, 1994). Thus, we assessed whether the constitutively-active ErbB4 mutants affected the growth rate or saturation density of FR3T3 fibroblasts. The FR3T3 cell lines described earlier were seeded in 60 mm dishes at a density of 2×10^4 cells per dish. Cells were incubated for 10 days to permit growth. During this period cells were counted every twenty-four hours.

The results of this experiment are shown in Figure 3.8. The growth rate of the cells that express ErbB2* is slightly greater than the growth rates of the other cell lines. Note that the growth rates of the cells that express the constitutively-active ErbB4 mutants are indistinguishable from the growth rates of cell lines that express wild-type ErbB4 or the vector control.

The saturation densities for the six cell lines are shown in Table 3.3. Again, note that the saturation density of the cell line that express ErbB2* is higher than the saturation densities of the other cell lines. Moreover, the saturation densities of the cell lines that express the ErbB4 mutants are not markedly higher than the saturation densities of the vector control cell line or the cell line that expresses wild-type ErbB4. Once again, these data suggest that constitutive ErbB4 signaling does not oncogenically transform the growth of fibroblasts.

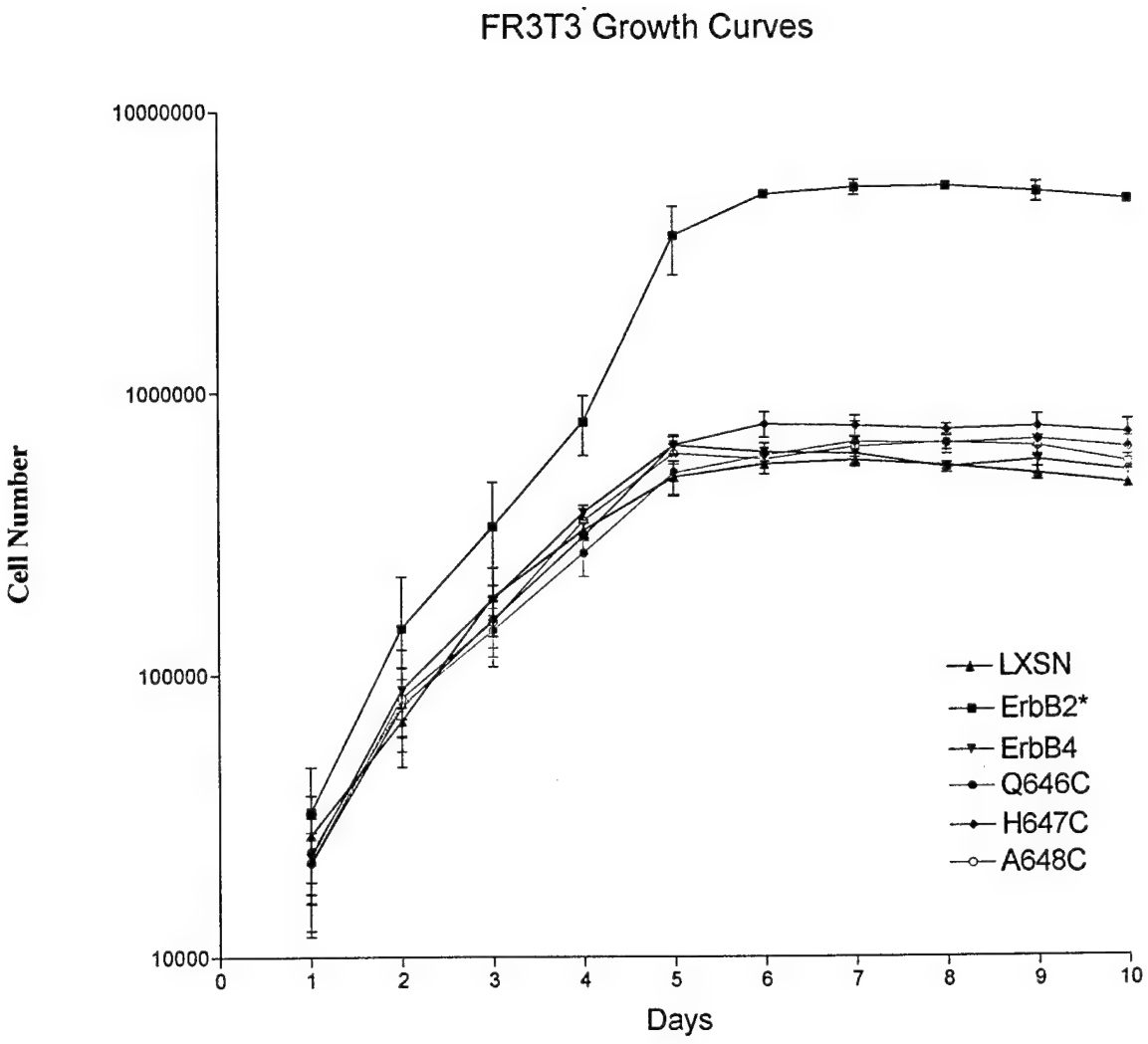

Figure 3.8 **Growth curves of constitutively-active ErbB4 mutant receptors parallel the wild-type ErbB4 receptor.** FR3T3 cells that express the vector control, ErbB2*, wild-type ErbB4, or the receptor mutants (Q646C, H647C, A648C) were plated at a density of 2 x 10^4 cells in 60 mm dishes and allowed to proliferate from one to ten days. Cells were counted daily to assess growth rates and saturation densities. The mean for three experiments is shown with error bars representing the standard error of the mean.

Table 3.3 **Saturation densities of FR3T3 cells expressing constitutively-active ErbB4 receptors**. FR3T3 cells infected with vector alone, ErbB2*, wild-type ErbB4, or the receptor mutants (Q646C, H647C, A648C) were plated at a density of 2×10^4 cells in 60 mm dishes and allowed to proliferate from one to ten days. Cells were counted daily to identify the saturation density for each cell line.

Saturation Densities	
LXSN	$5.8 \pm 0.3 \times 10^5$
ErbB2*	$5.4 \pm 0.1 \times 10^6$
ErbB4	$6.1 \pm 0.5 \times 10^5$
Q646C	$6.6 \pm 0.6 \times 10^5$
H647C	$7.6 \pm 0.7 \times 10^5$
A648C	$6.6 \pm 0.4 \times 10^5$

CHAPTER IV
DISCUSSION

In this report, we show that introducing a cysteine residue in the proximal region of ErbB4 causes constitutive dimerization and signaling by the receptor. We found that mutating any one of three different sites in the extracellular region of ErbB4 causes ligand-independent tyrosine phosphorylation and increased kinase activity. However, we were not able to show that the constitutively-active ErbB4 mutants induce increased proliferation, loss of contact inhibition, or anchorage-independent growth in fibroblasts. These results suggest that increased signaling by ErbB4 homodimers is not sufficient to promote tumorigenesis.

ErbB4 has not been shown to function independently in the progression to metastatic disease. Our results support this hypothesis. Unlike other ErbB family members, ErbB4 appears to be associated with differentiation. Multiple studies are in agreement with this conclusion. An agonistic anti-ErbB4 antibody induces differentiation of cultured MCF-7 breast cancer cells (Chen *et al.,* 1996). Neuregulin, a ligand for ErbB3 and ErbB4, induces branching and differentiation of the mammary epithelium into milk protein-secreting lobuloalveolar structures *in vivo* (Peles *et al.,* 1992; Jones *et al.,* 1996). Higher ErbB4 expression levels are associated with a more differentiated phenotype in breast cancer (Bacus *et al.,* 1996; Kew *et al.,* 2000). Furthermore, ErbB4 activation during late pregnancy is responsible for inducing Stat5 activity (an important mediator of differentiation in mammary epithelium), which regulates differentiation (Jones *et al.,* 1999).

While ErbB4 receptor expression or signaling is observed in a number of differentiated tissues, ErbB4 is overexpressed along with other ErbB family members in malignant tissues (Haugen, *et al.,* 1996; Gilbertson, *et al.,* 1997). ErbB1 or ErbB2

overexpression is associated with aggressive behavior in a wide range of solid tumors including tumors of the breast, ovary, and prostate (Hynes and Stern, 1994). ErbB3 is predominately overexpressed in a subset of human mammary and gastric cancers (Kraus *et al.,* 1989; Sanidas *et al.,* 1993). Finally, overexpression of ErbB4 is observed in a series of ovarian (up to 90%), childhood medulloblastoma, thyroid, breast, and gastric cancers (Gilbertson *et al.,* 1997; Haugen *et al.,* 1996; Kew *et al.,* 2000; Kato, 1998). From these observations we cannot rule out that increased ErbB4 signaling contributes to tumorigenesis or the malignant phenotype.

Indeed, the functional consequences of ErbB4 expression and signaling may depend on many factors. For instance, ErbB family members have dynamic patterns of expression in various organs and tissues and each ErbB family member may require complex interactions with other ErbB family members to specify a cellular response (Yarden and Sliwkowski, 2001). Thus, it is thought that the biological specificity of ErbB4 signaling is defined by the combination of both homo- and hetero-dimeric ErbB receptor complexes formed upon ligand binding (Ferguson et al., 2000). Depending on the cell type, any one of the four ErbB receptors may predominate in heterodimer complexes. Thus, ErbB4 may require heterodimerization with another ErbB family member or may require signaling by another ErbB family receptor to couple to tumorigenesis or the malignant phenotype.

Moreover, ErbB4 homodimers may couple to different cellular responses than do heterodimers of ErbB4. ErbB4 homodimers are biologically active (couple to downstream pathways) and represent naturally occurring signaling entities (Sweeney *et al.,* 2000). One caveat to our analysis of signaling by ErbB4 homodimers is that the biological responses associated with heterodimeric complexes are not seen. Indeed, multiple lines of evidence suggest that the oncogenic potential of ErbB4 is a result of heterodimeric and not homodimeric complexes (Gilbertson *et al.,* 1997). Moreover, malignant growth transformation by ErbB4 has been shown to require co-expression with other ErbB family members, as well as an ErbB receptor ligand (Cohen *et al.,* 1996b). Interestingly, cells that overexpress ErbB1 form tumors in nude mice, whereas co-expression of ErbB1 with ErbB2 in nude mice enhances tumor formation. In contrast,

cells that co-express ErbB4 with ErbB1 were unable to form tumors. In fact, ErbB4 co-expression with ErbB1 reduces the tumorigenic potential of ErbB1 alone (Cohen *et al.*, 1996b). Together, all of these results indicate that ErbB4 does not couple to metastatic disease, but may regulate the oncogenic potential of other ErbB family members.

A related question is whether the three constitutively-active ErbB4 mutants couple to different cellular responses. The simplest prediction is that the Q646C, H647C, and A648C mutants are tyrosine phosphorylated at different sites. Since each cysteine mutation is predicted to be on different face of the receptor, the alignment of a receptor monomer with another receptor monomer may be different for each mutant and may define which tyrosine residues are phosphorylated. Our results are consistent with this model for receptor signaling. Only three of the ErbB4 mutants that were constructed were effectively expressed and tyrosine phosphorylated in Ψ2 cells (data not shown). Dimerization experiments indicate that a cysteine mutation at amino acid 646 may cause receptors to be predominately expressed as dimers (Figure 3.3). A648C displays higher intrinsic kinase activity than Q646C and H647C (Figure 3.5 and Table 3.2). Furthermore, ErbB4 mutants are tyrosine phosphorylated at different levels in monomeric and dimeric states (Figure 3.2 and 3.4). Thus, each cysteine mutation may force the alignment of the kinase domain on one receptor with different phosphorylation sites on the other receptor. As a result, proper alignment of receptor interfaces, possible through disulfide bonds, may differentially phosphorylate tyrosine residues to couple to downstream pathways. Therefore, we purpose that the constitutively-active ErbB4 receptors are not generic in their signaling capabilities. Indeed, further investigation is still needed to identify potential phosphorylation sites responsible for ErbB4 activation.

We hypothesize that our mutants mimic the ligand-activated state of the receptor and are differentially tyrosine phosphorylated. Recent observations indicate that different ligands induce differential ErbB4 tyrosine phosphorylation (Sweeney *et al.*, 2000). Ligand discrimination by the ErbB4 receptor results in differential coupling to intracellular signaling pathways. For example, BTC and NRG1β preferentially stimulate the recruitment of Shc to ErbB4, whereas NRG1β and NRG2β stimulate the recruitment of p85 to ErbB4. In MDA-MB-468 cells, differentially spliced variant of neuregulin

(NRG1β and NRG2β) were found to elicit distinct phosphorylation patterns responsible for recruiting SH2 and PTB domain-containing proteins (Crovello *et al.,* 1998). Furthermore, different ligands may induce different oligomeric states of the receptor. NRG1β has been shown to induce the formation of ErbB4 oligomers that are larger than dimers (Ferguson *et al.,* 2000). These results indicate that ErbB4 ligands induce differential patterns of receptor phosphorylation and oligomeric states that are capable of activating different biological responses.

We believe that the ErbB4 mutants Q646C, H647C, and A648C provide powerful tools for assessing the biological roles played by activated ErbB4 receptors. Until now, receptor cross-talk through multiple combinatorial interaction between ErbB family members has been problematic in studying ErbB4 receptor function. The generation of constitutively-active ErbB4 mutants provides a ligand-independent mechanism for examining the biological responses of ErbB4 activation. More generally, our observations significantly modify the understanding of signaling through the ErbB4 receptor and point to a number of possible models for its function, namely, differentiation or tumor suppression. Unfortunately, we were not able to ascertain the biological roles played by ErbB4 in the context of ligand-independent activation. Indeed, multiple studies suggest that ErbB4 has functional roles in differentiation. Our work supports those suggestions and has laid the groundwork for further investigation of the physiological outcomes of ErbB4 signaling.

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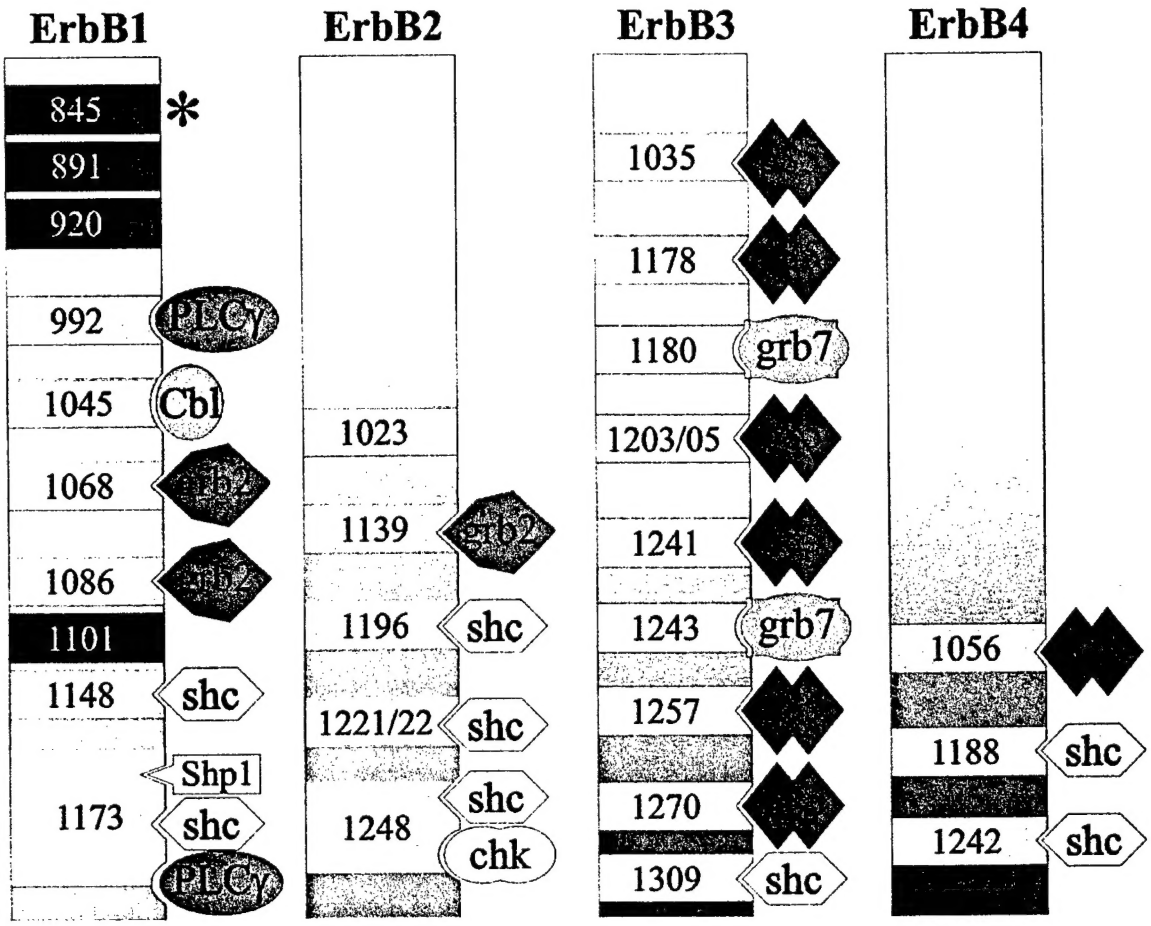
APPENDICES

Appendix A

Diagrammatic Representation of Specific Phosphotyrosine Residues and Signaling Molecules For The ErbB Family of Receptor Tyrosine Kinases

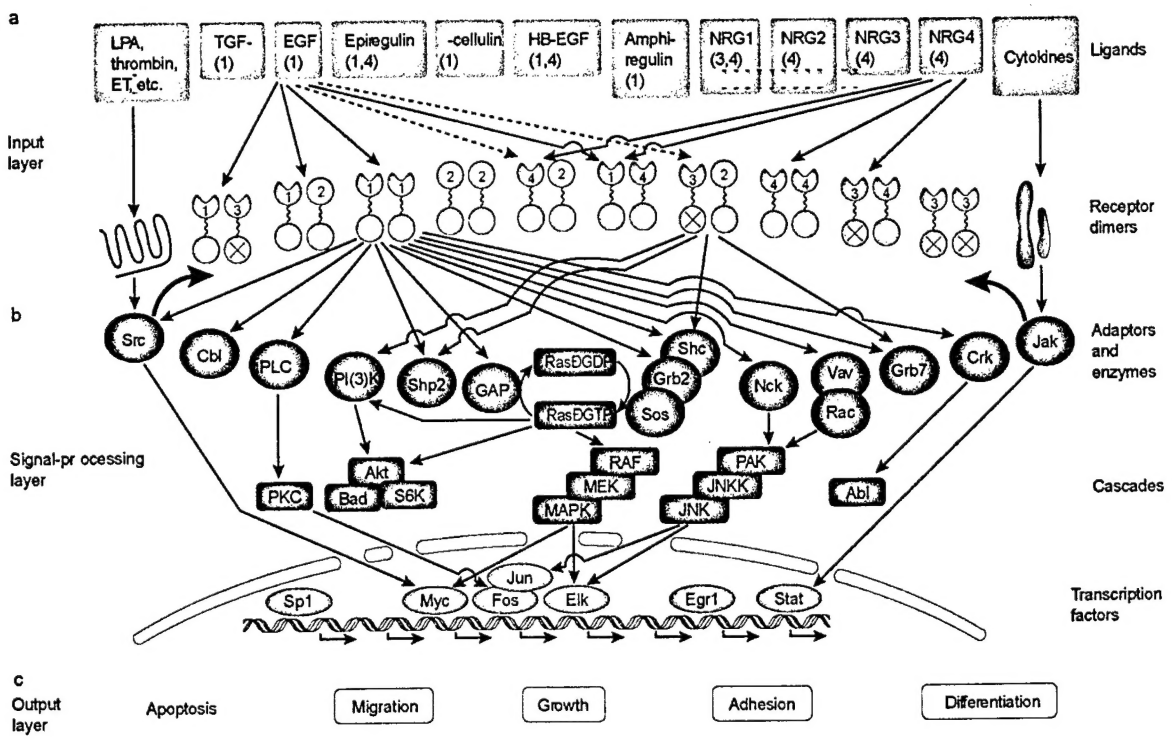


(Reproduced from Olayioye *et al.*, 2000)

Cytoplasmic domains of the four ErbB RTKs are shown. Tyrosine residues that have been identified as autophosphorylation sites are numbered and sites for the Src kinase are indicated in black. Adapter proteins are shown bond to their respective sites. The asterisk represents a tyrosine in the T loop of the kinase domain of ErbB1.

Appendix B

The ErbB Signaling Network

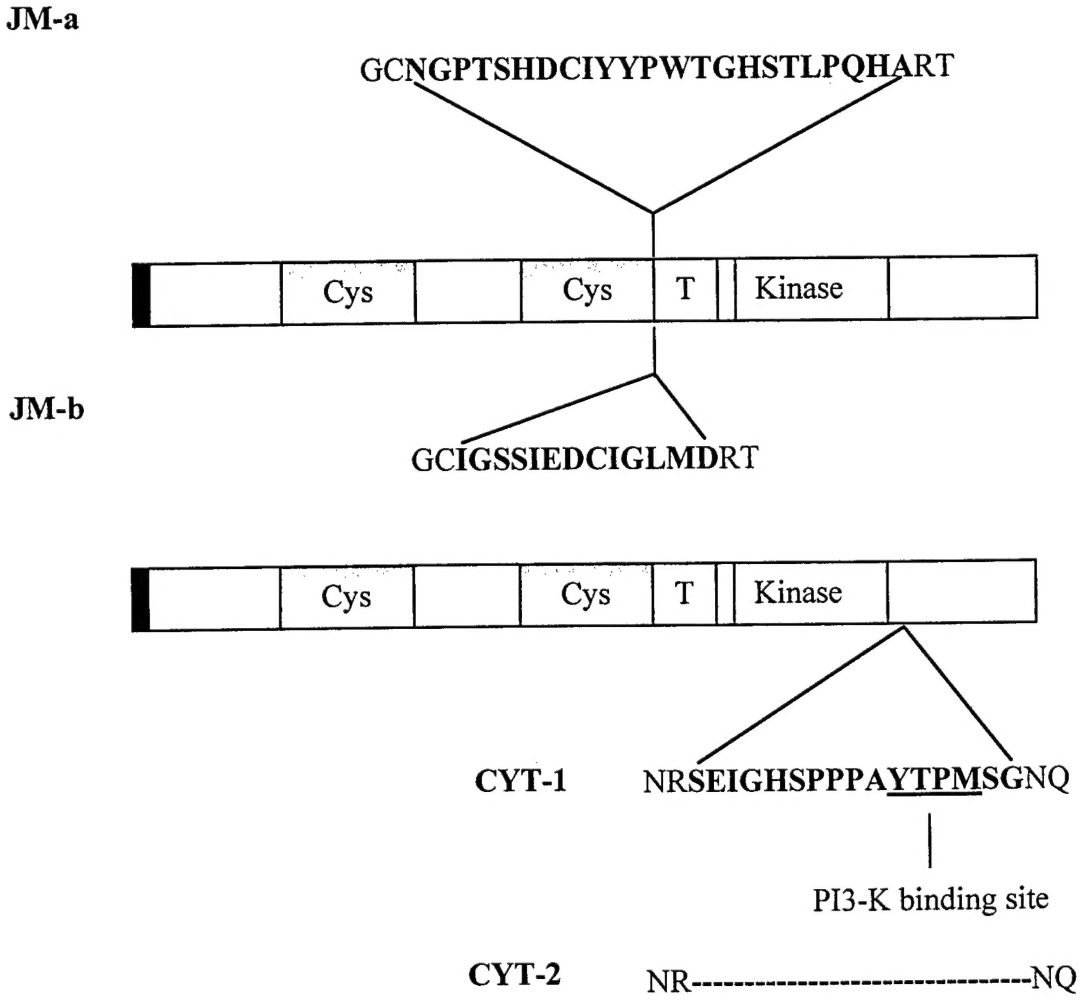


(Reproduced from Yarden and Sliwkowski, 2001)

a. Ligands and the ten dimeric receptor combinations comprise the input layer. Numbers in each ligand block indicate the respective high-affinity ErbB receptors. ErbB2 binds no ligand with high affinity, and ErbB3 homodimers are catalytically inactive (crossed kinase domains). **b.** Signaling to the adaptor layer, only some of the transcription factors are represented in this layer. **c.** Signaling to the output layer is poorly understood at present.

Appendix C

ErbB4 Isoforms



The deduced amino acid sequences of the alternative extracellular juxtamembrane (JM-a and JM-b) and cytoplasmic (CYT-1 and CYT-2) domains of the ErbB4 receptor. JM-a contains a 23 amino acid form, whereas JM-b contains a 13 amino acid form. CYT-1 contains the only PI3-K binding site in ErbB4, isoform CYT-2 does not. JM-a and CYT-1 are identical forms of the ErbB4 receptor. The alternative sequences are in boldface. *Cys*, cysteine-rich domains; *T*, transmembrane domain; *Kinase*, tyrosine kinase domain. (JM isoforms – Elenius *et al.,* 1997; CYT isoforms – Elenius *et al.,* 1999)